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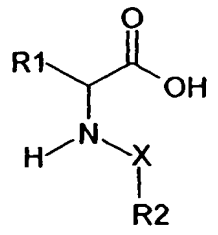
(54) Title: FMOC-L-LEUCINE AND DERIVATIVES THEREOF AS PPAR-γ AGONISTS



(I)

(57) Abstract: The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine or derivatives thereof, of the formula I. Said method is particularly useful for treating or preventing anorexia, hyperlypidemia, insulin resistance, inflammatory diseases, cancer and sking disorders.

FMOC-L-Leucine and derivatives thereof as PPARγ agonists

The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine (N-(9-fluroroenylmethyloxycarbonyl)-L-Leucine) or derivatives thereof.

The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors which bind DNA as heterodimers with the retinoid X receptor (RXR) and activate a number of target genes, mainly involved in the control of lipid metabolism. PPARs have pleiotropic biological activities and wide-ranging medical applications, ranging from uses in metabolic disorders to eventual applications in inflammation, and cancer (Desvergne and Wahli, 1999; Schoonjans *et al.*, 1997; Spiegelman and Flier, 1996). Especially, PPARγ has received a lot of attention because PPARγ-activating drugs represent a novel opportunity to treat type 2 diabetes. PPARγ can be activated by naturally occurring ligands, such as the long-chain fatty acid-derivatives, 15-deoxy-Δ12,14-prostaglandin J2, Δ12-prostaglandin J2 (PG J2), and 9- and 13-cis-hydroxyoctadecadienoic acid (HODE) (Forman *et al.*, 1995; Kliewer *et al.*, 1995; Nagy *et al.*, 1998). Most interesting is, however, the observation that the anti-diabetic activity of a group of the glitazones, which all possess a thiazolidinedione ring (Figure 1, Panel A), results from their PPARγ activating properties (Berger *et al.*, 1996; Willson *et al.*, 1996). The therapeutic efficacy of the current thiazolidinediones (TZDs) in type 2 diabetes is, however, far from optimal and several undesirable side-effects have been reported for this drug class (Schoonjans and Auwerx, 2000). As a result, there is a need for non-TZD-based alternative ligands of PPARγ.

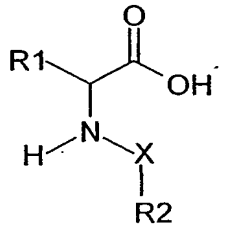
Recently, a series of L-tyrosine based PPARγ ligands were designed by replacing the thiazolidinedione ring with a carboxylic acid and by introducing an amine function on the adjacent carbon while keeping the parahydroxybenzyl sequence (Figure 1, Design 1 route). An optimal PPARγ activity was obtained when the amine on the alpha carbon of the L-tyrosine ligands was substituted with a benzoylphenyl function, leading to the development of *N*-(2-benzoylphenyl)-L-tyrosine derivatives (Figure 1, Panel B, left part) (Cobb *et al.*, 1998; Collins *et al.*, 1998). Rigidifying the benzoyl and phenyl moieties of this alpha-amino substituent through an additional phenyl-phenyl bond (Figure 1, Panel B, right part) leads to compounds with good potencies (Cobb *et al.*, 1998; Collins *et al.*, 1998).

In connection with the present invention, it was unexpectedly found that FMOC-L-tyrosine derivatives were devoid of PPARγ activity, whereas FMOC-L-leucine (hereafter also designated as F-L-Leu), whose structure is lacking the parahydroybenzyl sequence present in both the TZDs and previously developed L-tyrosine-based PPARγ ligands (Figure 1), is a new potent insulin-sensitizing compound with unique PPARγ-activating and -binding properties.

F-L-Leu, referred to as NPC 15199, has been described as a drug active in various inflammatory models through an unknown anti-inflammatory mechanism (Miller *et al.*, 1993) (Burch *et al.*, 1991). But, the present invention provides new applications of this compound and derivatives thereof as a PPARγ agonist.

Description

The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of a compound having the formula I:



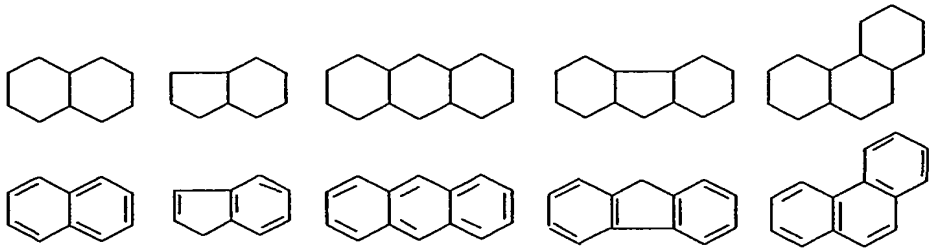
wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

X is a chain comprising from 1 to 6 carbon atoms which may comprise one to four heteroatoms,

R2 is a condensed polycyclic group comprising at least two cycles.

In a first embodiment, the R2 group comprises at least two cycles selected from carbocycles and heterocycles.

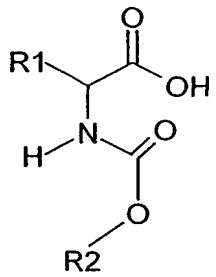
The R2 group can be advantageously selected from



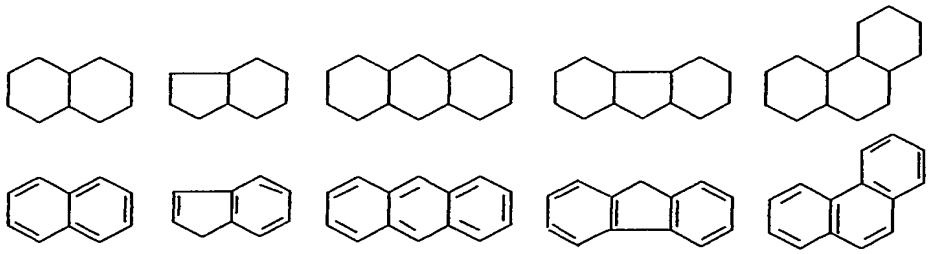
wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

In a second embodiment, the X chain comprises one or two carbon atoms which may be subtituted by an oxo group.

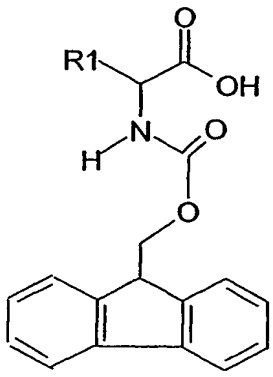
A preferred embodiment of the invention is directed to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,
R2 is a polycyclic group selected from

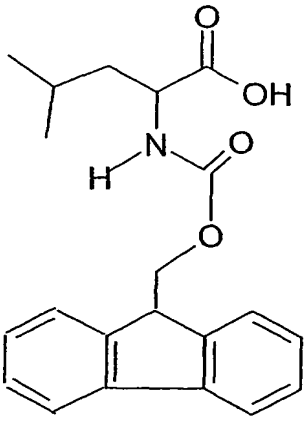


wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.
Compounds that are more particularly suitable have the formula :



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms and wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S.

For example, a preferred compound is



wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S; such as N-(9-fluroroenylmethyloxycarbonyl)-L-Leucine.

The method according to the invention is useful for treating or preventing anorexia, fo increasing or decreasing body weight, treating or preventing hyperlypidemia, fo increasing insulin sensitivity and for treating or preventing insulin resistance, as occur in diabetes.

Among the other diseases or conditions that can be treated or prevented with th compounds described above, chronic inflammatory disorders such as inflammator bowel disease, ulcerative colitis, Crohn's disease, arthritis, notably rheumatoi arthritis, polyarthritis and asthma are relevant.

The invention can also be reduced to practice for cancer, notably colon, prostate an hematological cancer, as well as for atherosclerosis and skin disorders, notabl psoriasis.

We tested a number of the FMOC-aminoacid series for PPARγ-binding and activation Interestingly, whereas FMOC-L-tyrosine, which was structurally most similar to the L tyrosine based PPARγ ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998), was devoid o PPARγ-activating properties, another member of the FMOC-aminoacid series, F-L-Le bound and activated PPARγ in a comprehensive set of *in vitro* and *in vivo* tests Evidence supporting FMOC-L-leucine as a stereoselective PPARγ agonist ligand i provided by the following arguments:

1) F-L-Leu binds *in vitro* to PPARγ as evidenced by ESI-mass spectrometry (figure 5 and protease protection assays (figure 4);
2) F-L-Leu enhanced co-activators recruitment to the PPARγ protein (figure 6);
3) F-L-Leu activates PPARγ in cotransfection studies (figure 3);
4) F-L-Leu induces adipocyte differentiation as judged by increased lipi accumulation and the induction of adipocyte target genes, such as LPL and aP (figure 7);

5) F-L-Leu acts as a potent insulin-sensitizing agent in both diabetic and mor interestingly also in non-diabetic murine models (figure 8);

6) Finally, like the TZDs ((Jiang *et al.*, 1998; Su *et al.*, 1999), F-L-Leu also ha significant anti-inflammatory activities and could prevent inflammatory bowe disease (figure 9). Since F-L-Leu is clearly structurally different fron thiazolidinediones and L-tyrosine based PPARγ ligands (Cobb *et al.*, 1998; Collin *et al.*, 1998) and since F-L-Leu presents little or no structural analogies with th partial agonists GW0072 (Oberfield *et al.*, 1999) and L-764406 (Elbrecht *et al.* 1999) and the antagonist BADGE (bisphenol A diglycidyl ether) (Wright *et al.* 2000), F-L-Leu defines a chemically new class of PPARγ ligands.

Although F-L-Leu shares several functional characteristics with known PPAR ligands, an important number of features distinguish F-L-Leu from these compounds which will be addressed hereinafter.

F-L-Leu possesses an acidic function with the ability to liberate a proton, provided b its carboxylic group. This is a feature shared by the natural ligand, PG J2, as well a previously developed L-tyrosine based ligands. Such an acidic function is also presen in the TZD ring at the level of the nitrogen located between the two carbonyl groups. A carboxylic group is also recovered in other PPARγ ligands such as GW0072, a weal partial agonist which antagonizes adipocyte differentiation, but in which lateral side chain substitution is approximately ten carbon atoms distant from the carboxylat (Oberfield *et al.*, 1999). This distance is in contrast with agonists such as the tyrosine derived ligands and F-L-Leu where a side-chain substitution occurs on the alpha-amin position. The stereoselectivity of the activation of PPARγ with the FMOC L- and D leucines (L- by far more potent than the D-enantiomer) confirms previous observation

made on other ligands with an asymmetric carbon including 8-HETE (S>R) and ar alpha-trifluoroethoxy-propanoic acid derivative (S>R) (Rangwala *et al.*, 1997; Young *et al.*, 1998; Yu *et al.*, 1995).

Experimental evidence suggests that F-L-Leu would interact with the ligand binding site of PPARγ in a fashion distinct from both the TZDs and the tyrosine-based ligands Nuclear receptors generally only will dock one ligand molecule in their ligand binding pocket, which fits rather tightly around the ligand. Binding of both TZDs and tyrosine-based PPARγ ligands follows this paradigm of "1 ligand/1 receptor" (Willson *et al.* 2000). The rather spacious ligand binding pocket of PPARγ, however, would not only allow the binding of large ligands, such as the tyrosine-based ligands, but eventually also allow binding of multiple ligand molecules to a single receptor. Our ESI-mass spectrometry data confirm that this is in fact the case with the F-L-Leu, where two molecules are shown to be bound to PPARγ ligand binding domain.

The capacity of PPARγ to bind two molecules of F-L-Leu could underly in fact some of the particular biological characteristics of this ligand. In fact, in transfection experiments F-L-Leu was two orders of magnitude less potent than the TZD rosiglitazone, although both compounds had similar maximal efficacy. The lower potency and the steep dose-response curve could therefore be explained by the fact that a higher molar ratio of ligand to receptor is required to change its configuration, a finding consistent both with the results of our protease protection (figure 4) and cofactor interaction assays (figure 6).

Although, less potent *in vitro*, F-L-Leu compares rather favorably to TZDs, such as rosiglitazone, for anti-diabetic activity *in vivo*. Administration of F-L-Leu (1

mg/kg/day) to the diabetic db/db mice improved insulin sensitivity more dramatically than an equivalent dose of rosiglitazone. This could be deduced from the more robust reduction of the AUC in IPGTT for an almost equivalent reduction in fasting insulin levels. Furthermore, at a dose of 30 mg/kg/day F-L-Leu was able to significantly improve insulin sensitivity in normal animals, an effect never observed with glitazones. One caveat with this comparison between the *in vivo* efficacy of F-L-Leu relative to the TZDs, lies in the intraperitoneal route of drug administration used here. This is the only route described at present to be effective for F-L-Leu, but it is known to be suboptimal for TZDs, which are readily orally bioavailable. Despite this potential draw-back, the results obtained with F-L-Leu as a potential anti-diabetic drug remain, however, remarkable. Moreover, F-L-Leu structure does not share a TZD ring, but offers a isosteric version of this chemical group via a carboxylic function (see figure 1), which is devoid of TZD-related side effects.

In summary, we describe F-L-Leu as a small synthetic PPARγ ligand. Unlike known PPARγ ligands, two molecules of F-L-Leu bind to a single PPARγ molecule, making its mode of receptor interaction novel and interesting. This unique way of receptor interaction, underlies some of the particular pharmacological properties of F-L-Leu. In general, F-L-Leu exerts similar biological activities as the known groups of PPARγ agonists, with a distinct pharmacology, characterized by a lower potency, but similar maximal efficacy. This novel synthetic molecule represents hence a new pharmacophore, which can be optimized according to routine procedures, for modulation of PPARγ biological activity.

Figure legends

Figure 1: Schematic representation of PPARγ ligand structures. The differen routes followed for the design are indicated.

A. anti-diabetic glitazones

B. L-tyrosine based PPARγ ligands

C. FMOC amino acids

Figure 2: Modulation of transcriptional activity of PPARγ2 by FMOC-amino-aci in Hep G2 cells. Hep G2 cells were co-transfected with an expression vector fo PPARγ2 (0.1 μg/well), pGL3-$(J_{wt})_3$TKLuc reporter construct (0.5 μg/well), an pCMV-βGal (0.5 μg/well), as a control of transfection efficiency (0.5 μg/well). The were then grown during 24 h in the presence or absence of indicated compound Activation is expressed as relative luciferase activity/β-galactosidase activity. Eac point was performed in triplicate. This figure is representative of three independen experiments.

Figure 3: F-L-Leu enhances transcriptional activity of PPARγ2 in different cel lines. RK13 cells **(A and D)**, CV1 cells **(B)** or Hep G2 cells **(C)** were co-transfecte with an expression vector for PPARγ2 (0.1 μg/well), pGL3-$(J_{wt})_3$TKLuc reporte construct (0.5 μg/well), and pCMV-βGal (0.5 μg/well), as a control of transfectio efficiency (0.5 μg/well). They were then grown during 24 h in the presence or absenc of indicated compound. Activation is expressed as relative luciferase activity/β galactosidase activity. Each point was performed in triplicate, and each figure i representative of four independent experiments.

Figure 4: F-L-Leu ligand alters the conformation of PPARγ. ^{35}S-PPARγ wa synthesized *in vitro* in a coupled transcription/translation system. Labeled PPARγ wa subsequently incubated with DMSO (0.1%), rosiglitazone (10^{-4}M) or F-L-Leu (10^{-4}M

followed by incubation with distilled water or increasing concentrations of trypsin. Digestion products were analyzed by SDS-PAGE followed by autoradiography. The migration of intact PPARγ is indicated and the asterisk indicates the 25-kDa resistant fragment of PPARγ.

Figure 5: Two molecules of F-L-Leu bind to a single PPARγ molecule. ESI-mass spectrometry analysis.

Figure 6: F-L-Leu enhances the interaction of PPARγ with p300. The purified his-tagPPARγ2$_{DE203-477}$ protein was incubated with purified p300Nt-GST protein and glutathione-Q-Sepharose beads in presence of DMSO (0.1%), rosiglitazone (10^{-4} M) or F-L-Leu (10^{-3} M). The beads were then washed and the samples separated on SDS-PAGE and blotted. The blot was developed with anti-histidine antibodies.

Figure 7: F-L-Leu enhances adipocyte differentiation. Confluent 3T3-L1 cells were incubated with 2 µM insulin, 1 µM dexamethasone, and 0.25 mM isobuthyl methyl xanthine for two days. Then, the cells were incubated in presence of DMSO (0.1 %), F-L-Leu (10^{-5} M) or rosiglitazone (10^{-7} M) for 4 days. **A:** RNA was isolated from 3T3-L1 cells after different times of differentiation induction. Blots were hybridized with 36B4 (to control for RNA loading); LPL or aP2 cDNAs. **B:** Cells were stained with Oil Red O after 6 days. LPL: lipoprotein lipase.

Figure 8: F-L-Leu improves insulin sensitivity in C57BL/6j and db/db mice. Intraperitoneal glucose tolerance test (IPGTT) in C57BL/6j **(A)** or db/db **(B)** mice 10 to 12 weeks old (n=8). Diamonds correspond to DMSO-treated mice; squares to F-L-Leu-treated mice at the concentration of 10 mg/kg/day and triangles to F-L-Leu-treated mice at the concentration of 30 mg/kg/day (for C57BL/6j mice, A) or rosiglitazone-treated mice at the concentration of 10 mg/kg/day (for db/db mice, B). Insulinemia **(C)** and body weights **(D)** of db/db mice treated with DMSO, F-L-Leu (10 mg/kg/day) or rosiglitazone (10 mg/kg/day).

Figure 9: F-L-Leu protects against colon inflammation in TNBS-treated Balb/c mice. A: Ameho histologic scores (left panel) and survival rate (right panel) in TNBS-treated mice injected either with DMSO or F-L-Leu (50 mg/kg/day). **B:** TNFα and IL-1β mRNA levels in the colon of TNBS-treated mice injected with DMSO or F-L-Leu (50 mg/kg/day). Results are expressed as mean ± SEM.

The following materials and methods were used to perform the examples below.

Materials and methods

FMOC-derivatives were acquired at Sen Chemicals (Dielsdorf, Switzerland). Rosiglitazone and pioglitazone were kind gifts of Dr. R. Heyman (Ligand Pharmaceuticals, San Diego, CA). The antibodies directed against the AB domain of PPARγ were produced in our laboratory (Fajas *et al.*, 1997). The protease inhibitor cocktail was purchased at ICN (Orsay, France).

Cell culture and transient transfection assays

The CV1, RK-13, and Hep G2 cell lines were obtained from ATCC (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics. Transfections with chloramphenicol acetyltransferase (CAT) or luciferase (luc.) reporter constructs were carried out exactly as described previously (Schoonjans *et al.*, 1996). The pGL3-$(J_{wt})_3$TKLuc and the pGL3-$(J_{wt})_3$TKCAT reporter constructs contain both three tandem repeats of the J site of the apolipoprotein A-II promoter cloned upstream of the herpes simplex virus thymidine kinase (TK) promoter and the luciferase or the CAT reporter genes respectively (Vu-Dac *et al.*, 1995). The following

expression vectors were used; pSG5-hPPARγ2, a construct containing the entire cDNA of the human PPARγ2 (hPPARγ2) (Fajas *et al.*, 1997); pSG5-mPPARα (Isseman *et al.*, 1993); and pCMV-βGal, as a control of transfection efficiency.

Production of proteins and mass spectrometry

The p300Nt-GST, fusion protein was generated by cloning the N-terminal part of the p300 protein (a.a. 2 to 516) downstream of the glutathione-S-transferase (GST) protein in the pGex-T1 vector (Pharmacia, Orsay, France). The fusion proteins were then expressed in *Escherichia coli* and purified on a glutathione affinity matrix (Pharmacia). Human PPARγ (aa. 203 to 477 of PPARγ) was subcloned into the pET15b (Novagen, Madison, WI) expression vector. The his-tagPPARγ2$_{DE203-477}$ proteins were produced as follow. The protein was purified using a metal chelate affinity column with an affinity column Co^{2+} coupled agarose (High Trap chelatin, Pharmacia). The protein was eluted with 20mM Tris-HCl, 500mM NaCl, 130mM imidazole and 1-2 propanediol 2.5% (pH 8.5). A second purification step was made by gel filtration (Superdex 200 16/60, Pharmacia). The protein was eluted with 20mM Tris-HCl, 100mM NaCl, 5mM DTT and 1-2 propanediol 2,5% (pH 8.5).

Liquid chromatography-electrospray ionization (ESI)-mass spectrometry analysis was performed as previously described (Rogniaux *et al.*, 1999).

Protease protection and pull-down experiments

Protease protection experiments. The pSG5-hPPARγ2 plasmid was used to synthesize ^{35}S-radiolabeled PPARγ in a coupled transcription/translation system according to the protocol of the manufacturer (Promega, Madison, WI). The transcription/translation reactions were subsequently aliquoted into 22.5 µl and 2,5 µl of phosphate buffered saline +/- compound were added. The mixture was separated into 4.5 µl aliquots and 0.5 µl of distilled water or distilled water-solubilized trypsin were added. The protease

digestion were allowed to proceed for 10 min at 25°C and terminated by the additio denaturing loading buffer. After separation of the digestion products in a gel SDS PAGE 12% acrylamide, the gel was fixed in 10% acetic acid (v/v): 30% ethanol (v/v for 30 min, treated in Amplify™ (Amersham, Orsay, France) and dried. Th radiolabeled digestion products were visualized by autoradiography.

Pull-down experiments. The purified his-tagPPARγ DE proteins were incubated 1 hou at 22°C in pull-down buffer (phosphate-buffered saline 1x, Glycerol 10%, NP40 0,5% with either GST or p300Nt-GST fusion protein, glutathione-Q sepharose beads, and F L-Leu (10^{-3}M) or rosiglitazone (10^{-4}M) when necessary. The beads were then washe 4 times in pull-down buffer and boiled in 2x sample buffer. The samples wer separated by 12% acrylamide SDS-PAGE and transferred to nitrocellulose membrane: Blots were developed with antibodies directed against polyhistidine aminoaci sequences.

Adipocyte differentiation

3T3-L1 cells (ATCC, Rockville, MD) were grown to confluence in medium . (Dulbecco's modified Eagle's Medium with 10% fetal calf serum, 100 units/n penicillin, and 100µg/ml streptomycin). Confluent cells were incubated in medium . containing 2 µM insulin, 1 µM dexamethasone, and 0.25 mM isobuthyl meth xanthine for two days. Then, the cells were incubated in medium A in presence (absence of PPARγ agonist for 4 days, changing the medium every 2 day Adipogenesis was evaluated by analysis of the expression of adipocyte-specif markers and by staining of lipids with Oil Red O (Chawla and Lazar, 1994).

RNA preparation and analysis

RNA was isolated from 3T3-L1 cells by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Northern blot analysis of total cellular RNA was performed as described (Auwerx *et al.*, 1989) Lipoprotein lipase (LPL), aP2 and 36B4 were used as probes (Graves *et al.*, 1992 Laborda, 1991; Lefebvre *et al.*, 1997). For RT-competitive PCR, total RNA (5-10µg was reverse transcribed into complementary DNA (cDNA) (Desreumaux *et al.*, 1999 Fajas *et al.*, 1997). The RT reaction mixture was amplified by PCR using sense and antisense primers specific for β-actin, TNFα and IL-1β. The samples were subjected to 40 PCR cycles, consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 52-58°C, and primer extension for 1.5 min at 72°C using a Gene Amp PCR System 9700 (Perkin-Elmer Corporation, Foster City, CA). The quantity of mRNA was expressed as the number of TNFα or IL-1β cDNA per β-actin cDNA molecules.

Animal experiments, glucose metabolism and inflammation

All mice were maintained in a temperature-controlled (25 °C) facility with a strict 12 light/dark cycle and were given free access to food (standard mice chow; DO4, UAR France) and water. Animals received F-L-Leu or rosiglitazone by intraperitoneal injection.

C57Bl/6J and db/db mice (8 per group) were obtained through the Janvier laboratories (Laval-Le Genest, France). Intraperitoneal glucose tolerance tests (IPGTT) were performed as described (Kaku *et al.*, 1988). Briefly, mice were fasted overnight (18h and injected intraperitonealy (i.p.) with 25 % glucose in sterile saline (0.9 % NaCl) at a dose of 2 g glucose/kg body weight. Blood was subsequently collected from the tail for glucose quantification with the Maxi Kit Glucometer 4 (Bayer Diagnostic, Puteaux France) prior to and at indicated times after injection. Blood for insulin measurement

was collected in fasting mice from the retroorbital sinus plexus under chloroform anesthesia. Plasma was separated and insulin measured using a radio immunoassay kit (Cis bio international, Gif-sur-Yvette, France).

Male Balb/c mice (8 per group) were used for the colitis studies (Jackson laboratories, Bar Harbor, Maine). Colitis was induced by administration of 40 µl of a solution of TNBS (150 mg/kg, Fluka, Saint Quentin Fallavier, France) dissolved in NaCl 0.9% and mixed with an equal volume of ethanol (50% ethanol). This solution was administered intrarectally via a 3.5 F catheter (Ref EO 3416-1, Biotrol, Chelles, France) inserted 4 cm proximal to the anus in anesthesized mice [Xylasine (50 mg/kg of Rompun® 2%, Bayer Pharma, Puteaux, France) and Ketamine (50 mg/kg of Imalgene® 1000, Rhône Mérieux, France)]. Animals were sacrified by cervical dislocation under ether anesthesia two days after TNBS administration. The colon was quickly removed, opened, washed. A 2 cm colonic specimen located precisely 2 cm above the anal canal was dissected systematically in 4 parts. One part was fixed overnight in 4% paraformaldehyde acid at 4°C, dehydrated in alcohol and embedded in paraffin. Sections (5 µm) were then deparaffined with xylene and rehydrated by ethanol treatment. Stained sections with haematoxylin-eosin were examined blindly by a pathologist and scored according to the Ameho criteria (Ameho *et al.*, 1997). The other parts of the colon were used for RNA isolation for the quantification of TNFα and IL1β mRNA expression.

Statistical analysis

Values were reported as mean +/- standard deviation. Statistical differences were determined by the Mann-Whitney U test. $P<0.05$ was accepted as statistically significant.

Example 1 : FMOC-L-leucine activates PPARγ in cell transfection experiments

Various FMOC derivatives of unsubstituted (L-tyrosine, D-leucine, and L-leucine) aminoacids were tested and compared to rosiglitazone or pioglitazone (as positive internal controls) for their ability to activate PPARγ in transient transfection experiments in HepG2 cells using the pSG5-hPPARγ2 expression and J_3TKpGl_3 reporter plasmids. In contrast to L-tyrosine PPARγ ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998), the FMOC substituted L-tyrosine derivative did not activate PPARγ. Significant PPARγ activity could, however, be detected for F-L-leu at the concentration of 10^{-5} M (figure 2). In contrast, no significant PPARγ activation was detected with the FMOC-D-leucine derivative, demonstrating that PPARγ activating properties of F-L-leu were stereoselective. Additional transfection experiments with F-L-Leu were performed on different cell lines (RK13, CV1 and HepG2 cells) (figure 3 A, B and C). In the rabbit kidney RK13 cells, we found that rosiglitazone has an optimal activity between 10^{-8} to 10^{-7} M. For F-L-Leu, PPARγ activation occurred at concentrations of 10^{-5} M (figure 3A). Consistent with previous results, F-L-Leu concentrations of 10^{-5} M were also required for optimal PPARγ activation in simian renal cells CV1 (figure 3B), and in human HepG2 cells (figure 3C). The optimal concentration for PPARγ activation by F-L-Leu was similar to that of PG J2 and 100-fold higher than the concentration of rosiglitazone (figure 3C) or pioglitazone (data not shown) necessary to reach the same efficacy.

Finally, we tested whether FMOC-amino acid derivatives synergized or antagonized rosiglitazone activation of PPARγ in RK13 cells (figure 3D). No significant modification of PPARγ activity was observed when we added either F-L-Leu, FMOC L-tyrosine or FMOC D-leucine (10^{-5} M) to a saturating concentration of rosiglitazone.

These results furthermore confirmed (see figure 3A) that we reached maximal PPARγ activation using rosiglitazone and F-L-Leu at the concentration of 10^{-7}M and 10^{-5}M respectively.

Example 2: FMOC L-leucine changes PPARγ conformation

Thiazolidinediones can induce an alteration in the conformation of PPARγ, as assessed by generation of protease-resistant bands following partial trypsin digestion of recombinant receptor (Berger *et al.*, 1999; Elbrecht *et al.*, 1999). Upon incubation of rosiglitazone with PPARγ, a fragment of approximately 25 kDa is protected from trypsin digestion whereas no protection is detected when PPARγ is incubated with DMSO vehicle (figure 4). Interestingly, F-L-Leu produced a protease protection pattern similarly to rosiglitazone, demonstrating that F-L-Leu altered PPARγ conformation (figure 4).

Example 3: Two molecules of FMOC-L-leucine interact with PPARγ

Electrospray ionization (ESI) mass spectrometry of hPPARγ LBD (amino acid 203 to 477) was used to identify the specific binding of F-L-Leu with PPARγ (figure 5). The purified fragment of PPARγ LBD was incubated with vehicle alone or either 1 or equivalents of F-L-Leu per equivalent of PPARγ. The mass of the receptor was determined after incubation by ESI-mass spectrometry. At 1 equivalent of F-L-Leu per equivalent of PPARγ, we could distinguish three populations of PPARγ corresponding to: 1/ unliganded PPARγ; 2/ a complex formed by 1 PPARγ LBD molecule and 1 F-L-Leu molecule; and 3/ a complex formed by 1 PPARγ LBD molecule and 2 F-L-Leu molecules. Interestingly, when we increased the F-L-Leu concentration (8 equivalents of F-L-Leu per 1 equivalent of PPARγ), we detected only the complex correspondi

to the PPARγ LBD bound with 2 F-L-Leu molecules. These results indicate that two molecules of F-L-Leu interact with one molecule of the PPARγ in a highly specific manner.

Example 4: FMOC-L-leucine enhances PPARγ/p300 interaction

PPARγ has been previously reported to interact with the cofactor p300. The overall molecular PPARγ/p300 interaction was the resultant of a ligand-independent binding of p300 to PPARγs' ABC domain and a ligand-dependent interaction of p300 with the PPARγ DE domains (Gelman *et al.*, 1999). Hence the purified PPARγ DE protein represents a tool to study the efficacy of PPARγ ligand binding properties in view of its' ability to recruit p300 upon ligand binding. Compared to the DMSO control, both rosiglitazone and F-L-Leu effectively induced the formation of PPARγ DE/p300Nt-GST complexes. This confirms that the F-L-Leu is a PPARγ ligand and that its binding to the PPARγ DE domain is capable of inducing conformational changes required for association with p300. The potency of the F-L-Leu compound was in this assay 2- to 3-fold lower than that of rosiglitazone.

Example 5: FMOC-L-leucine induces adipocyte differentiation

The ability of F-L-Leu and rosiglitazone to stimulate adipocyte differentiation of murine pre-adipocyte 3T3-L1 cells were next compared. Adipogenesis was monitored by analysis of lipoprotein lipase (LPL) and aP2 mRNA levels as markers of adipocyte differentiation and by studying morphological changes associated with the differentiation process. F-L-Leu at the concentration of 10^{-5} M significantly stimulated both LPL and aP2 mRNA levels to an extent close to that seen in cells incubated with

rosiglitazone at the concentration of 10^{-7} M (figure 7A). Staining of 3T3-L1 cells with Oil Red O, as a marker for neutral lipid accumulation, was performed after a 6 days incubation of cells with either DMSO, or the two PPARγ ligands F-L-leu or rosiglitazone (Figure7B). The two drugs were again capable of inducing neutral lipid accumulation. Hence, like rosiglitazone, F-L-Leu was an adipogenic drug in 3T3-L1 cells.

Example 6: FMOC-L-leucine improves insulin sensitivity *in vivo*

To assess whether F-L-Leu could improve insulin sensitivity, we compared the glucose tolerance in C57BL/6j mice treated with F-L-Leu relative to that observed in control animals which received only the vehicle, DMSO (figure 8A). Mice were treated with 2 different doses of F-L-Leu (10 and 30 mg/kg/days) during 7 days and then IPGTT was performed. Intra-peritonealy administrated glucose was cleared in a comparable rate in mice receiving vehicle or F-L-Leu at 10 mg/kg/day. In mice treated with F-L-Leu at 30 mg/kg/day, the maximum glucose levels increased only to 320 mg/dl whereas the glucose levels climbed to 440 mg/dl after glucose injection for both 10 mg/kg/day F-L-Leu and the control group. Furthermore, the area under the curve was significantly lower in mice treated with F-L-Leu at 30 mg/kg/day relative to either control mice or mice receiving F-L-Leu at lower dose.

We next compared glucose tolerance in db/db mice treated with DMSO, F-L-Leu (10 mg/kg/day) or rosiglitazone (10 mg/kg/day) during 7 days. In control mice (DMSO group), glycemia rapidly increased after glucose loading, reaching a maximum of 500 mg/dl between 45 to 60 min after injection, before slowly decreasing. In rosiglitazone-treated mice, glucose loading was better "tolerated" than in control animals with a

reduction in the maximal glycemia (350 mg/dl), and a more rapid recovery of these supranormal values. F-L-Leu-treated animals showed the best glucose tolerance test, with a maximal glucose level (420 mg/dl) 20 min after injection and an immediate and fast subsequent decrease to normal (100 mg/dl) values within 120 min. Furthermore, 7 days treatment of animals with F-L-Leu and rosiglitazone resulted in a dose-dependent lowering of fasting serum insulin levels (mean values of 70 µUI/mL for db/db mice treated with either F-L-Leu or rosiglitazone versus 180 µUI/mL for the DMSO group) (figure 8C). These data clearly show that F-L-Leu improves insulin sensitivity in both diabetic and normal mice. Interestingly, whereas rosiglitazone had a tendency to increase body weight of mice, no difference in body weight was seen in mice treated with F-L-Leu during 8 days when compared to control mice (figure 8D). In addition, we observed a tendency to diminution of the liver weight for F-L-Leu-treated mice relative to control or rosiglitazone-treated mice (data not shown).

Example 7: FMOC-L-leucine protects against colitis

Intrarectal administration of TNBS has been shown to induce rapidly and reproducibly a colitis in mice as a result of covalent binding of TNP residues to autologuous host proteins leading to a mucosal infiltration by polynuclear cells, the production of TNFα , and the activation of NFκB (Allgayer *et al.*, 1989; Stenson *et al.*, 1992; Su *et al.*, 1999).We determined the survival rate and scored the colon damage as well as the production of cytokines two days after intra-rectal TNBS administration in control animals or animals which were treated 4 days with F-L-Leu at 50 mg/kg/day (figure 9). Interestingly, 100% of F-L-Leu-treated mice survived colon inflammation whereas only 76 % of control mice were alive after induction of inflammation. Administration of F-L-Leu furthermore reduced significantly the histologic score indicating that F-L-

Leu reduces ulceration, erosion and necrosis induced by inflammation. Finally, F-L-Leu administration resulted in a significant decrease in the mRNA levels expression of the pro-inflammatory cytokines, TNFα and IL-1β suggesting that, like with rosiglitazone, PPARγ activation by F-L-Leu protects against colon inflammation by inhibition of the TNFα signaling pathway.

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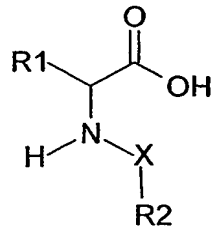
CLAIMS

1. A method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of a compound having the formula I:



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

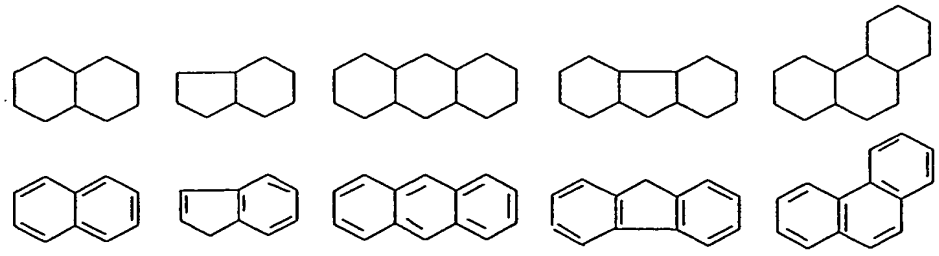
X is a chain comprising from 1 to 6 carbon atoms which may comprise one to four heteroatoms,

R2 is a condensed polycyclic group comprising at least two cycles.

2. A method according to claim 1 wherein the R2 group comprises at least two cycles selected from carbocycles and heterocycles.

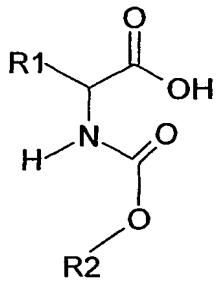
3. A method according to one of claims 1 and 2 wherein the X chain comprises one or two carbon atoms which may be substituted by an oxo group.

4. A method according to one of claims 1 to 3 wherein R2 is a polycyclic group selected from



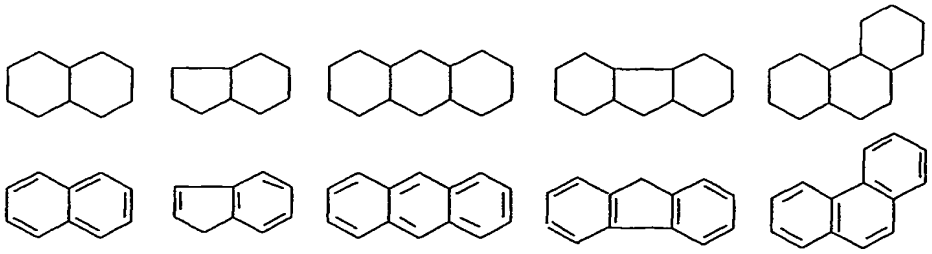
wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

5. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



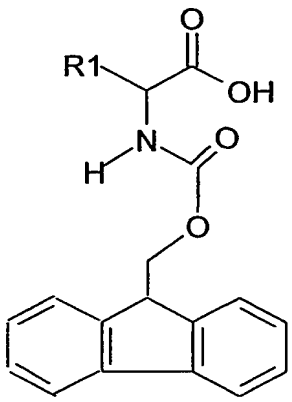
wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

R2 is a polycyclic group selected from



wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

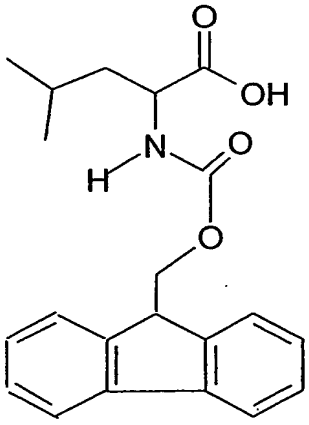
6. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms and

wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S

7. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S

8. A method according to claim 1 comprising administration of a therapeutically effective amount of N-(9-fluroroenylmethyloxycarbonyl)-L-Leucine.

9. A method according to one of claims 1 to 8 wherein said disease or condition is anorexia.

10. A method according to one of claims 1 to 8 for increasing or decreasing body weight.

11. A method according to one of claims 1 to 8 for increasing insulin sensitivity.

12. A method according to one of claims 1 to 8 for treating or preventing insulin resistance, as occurs in diabetes.

13. A method according to one of claims 1 to 8 wherein said disease or condition is a chronic inflammatory disorder.

14. A method according to one of claims 1 to 8 wherein said disease or condition is inflammatory bowel disease, ulcerative colitis or Crohn's disease.

15. A method according to one of claims 1 to 8 wherein the said disease or condition is arthritis, notably rheumatoid arthritis, polyarthritis and asthma.

16. A method according to one of claims 1 to 8 wherein said disease is cancer.

17. A method according to one of claims 1 to 8 wherein said disease is atherosclerosis.

18. A method according to one of claims 1 to 8 wherein said disease is a skin disorder, notably psoriasis.

19. A method according to one of claims 1 to 8 wherein said disease is hyperlypidemia.

Figure 1B

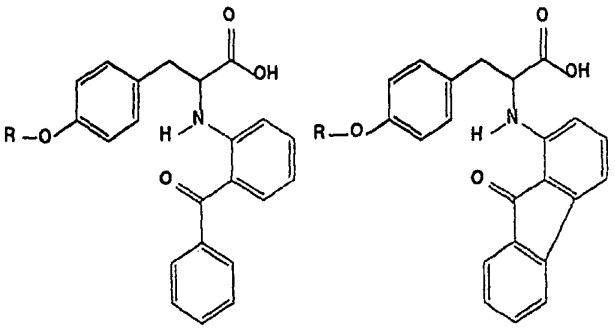
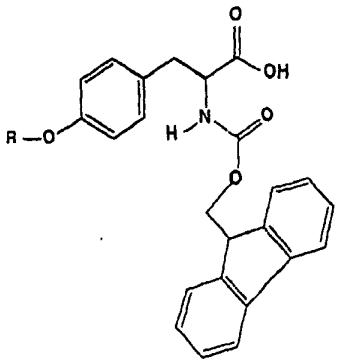
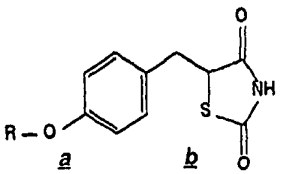
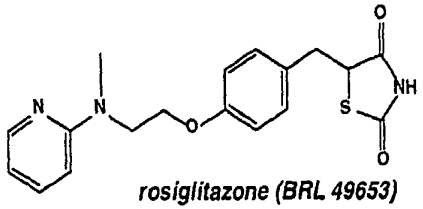
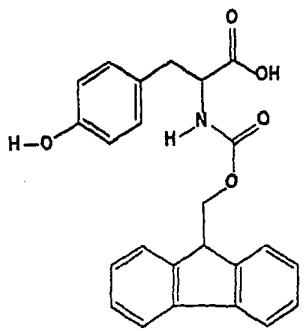


Figure 1C









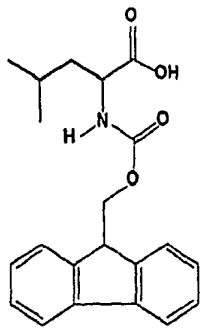


Figure 1A

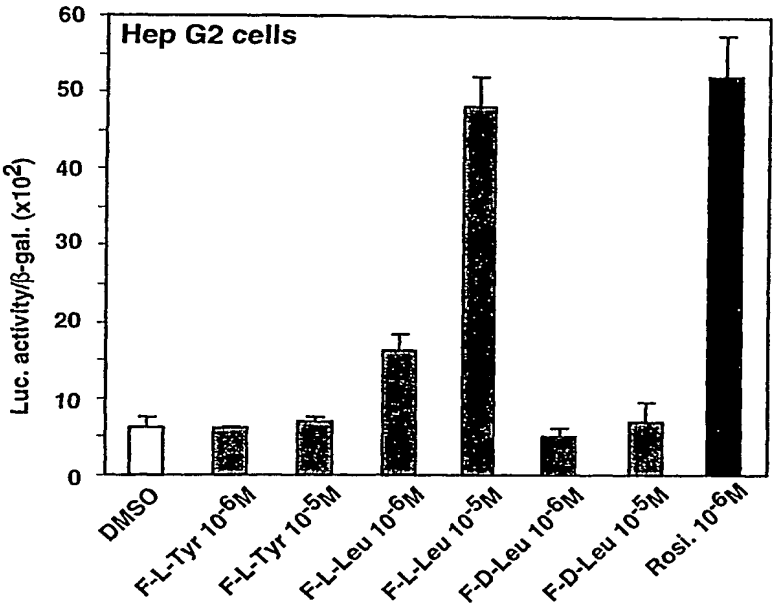

Hep G2 cells
Luc. activity/β-gal. (x10²)
60
50
40
30
20
10
0
DMSO
F-L-Tyr 10-6M
F-L-Tyr 10-5M
F-L-Leu 10-6M
F-L-Leu 10-5M
F-D-Leu 10-6M
F-D-Leu 10-5M
Rosi. 10-6M

Figure 2

Figure 3A

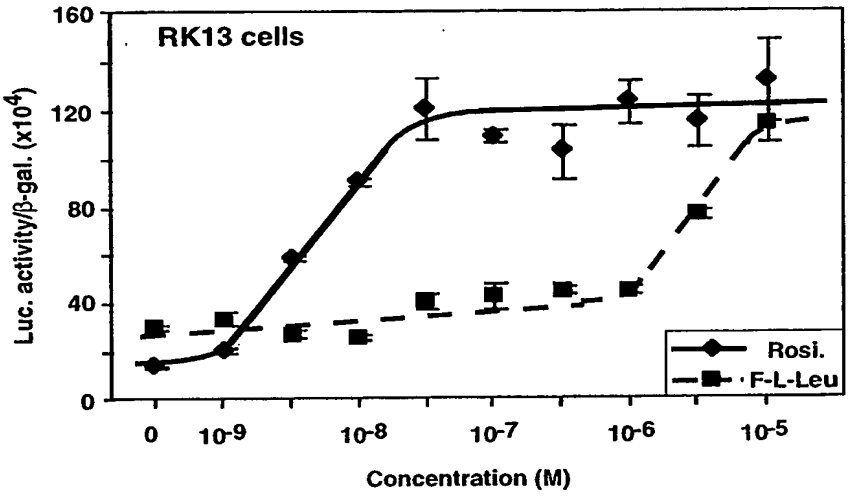


Figure 3B

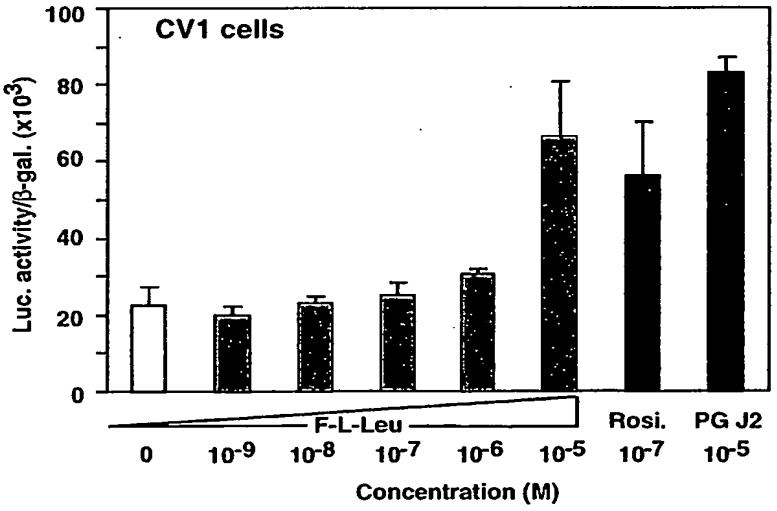


Figure 3C

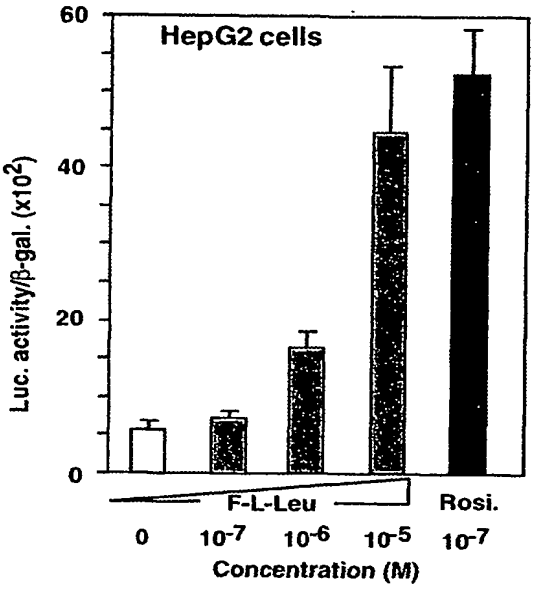
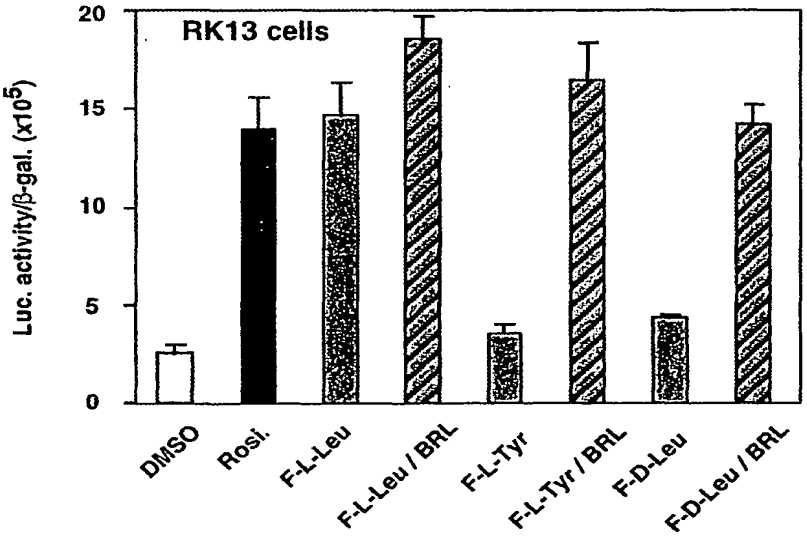


Figure 3D



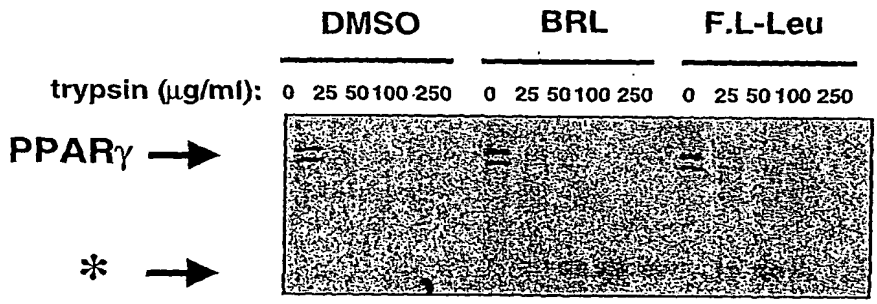

DMSO
BRL
F.L-Leu
trypsin (μg/ml): 0 25 50 100 250 0 25 50 100 250 0 25 50 100 250
PPARγ
*

Figure 4

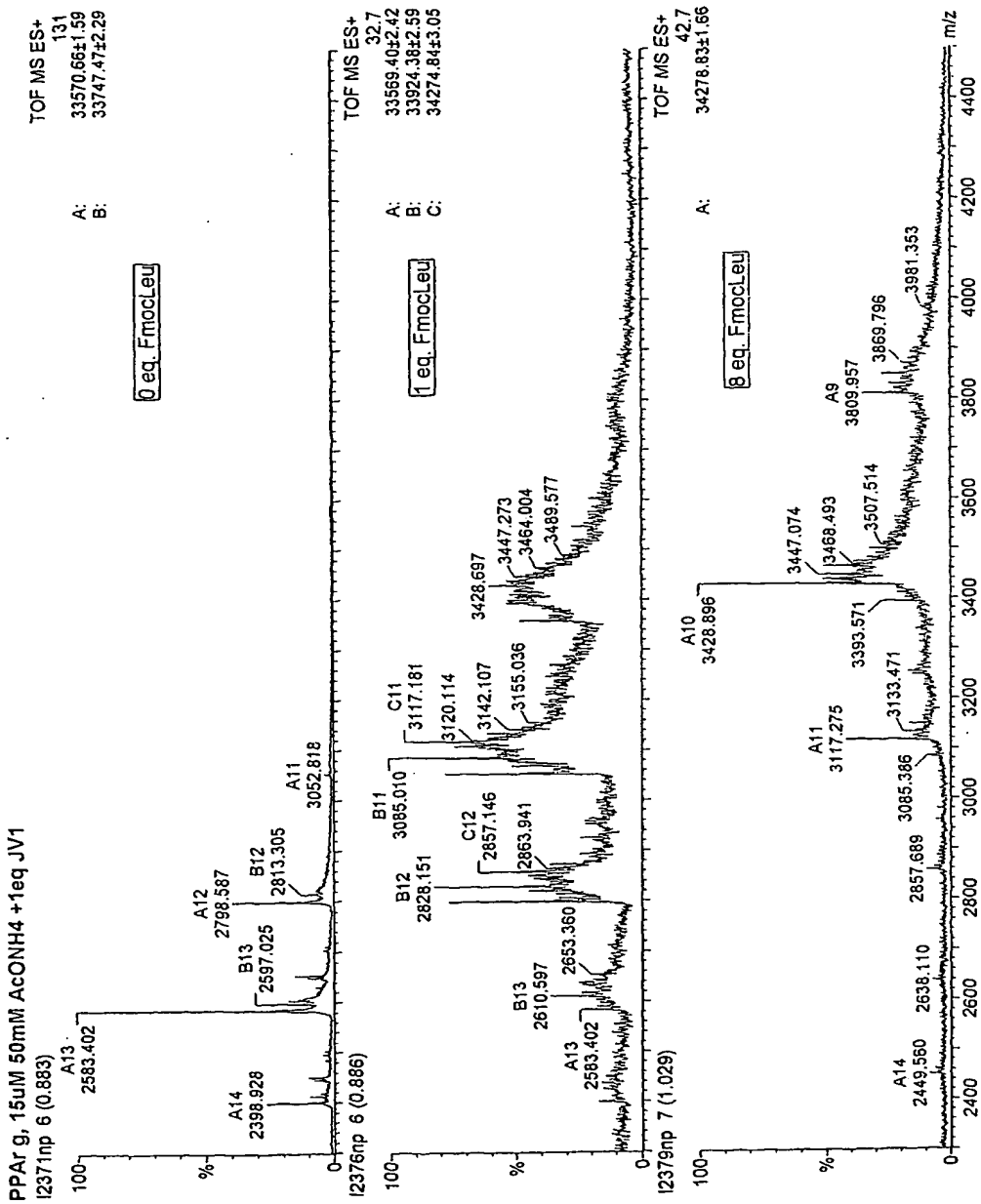

PPAr g, 15uM 50mM AcONH4 +1eq JV1
I2371np 6 (0.883)
TOF MS ES+
131
A: 33570.66±1.59
B: 33747.47±2.29
0 eq. FmocLeu
A13 2583.402
A14 2398.928
B13 2597.025
A12 2798.587
B12 2813.305
A11 3052.818
I2376np 6 (0.886)
TOF MS ES+
32.7
A: 33569.40±2.42
B: 33924.38±2.59
C: 34274.84±3.05
1 eq. FmocLeu
A13 2583.402
B13 2610.597
2653.360
B12 2828.151
C12 2857.146
2863.941
B11 3085.010
C11 3117.181
3120.114
3142.107
3155.036
3428.697
3447.273
3464.004
3489.577
I2379np 7 (1.029)
TOF MS ES+
42.7
A: 34278.83±1.66
8 eq. FmocLeu
A14 2449.560
2638.110
2857.689
3085.386
A11 3117.275
3133.471
3393.571
A10 3428.896
3447.074
3468.493
3507.514
A9 3809.957
3869.796
3981.353
%
100
0
2400
2600
2800
3000
3200
3400
3600
3800
4000
4200
4400
m/z

Figure 5

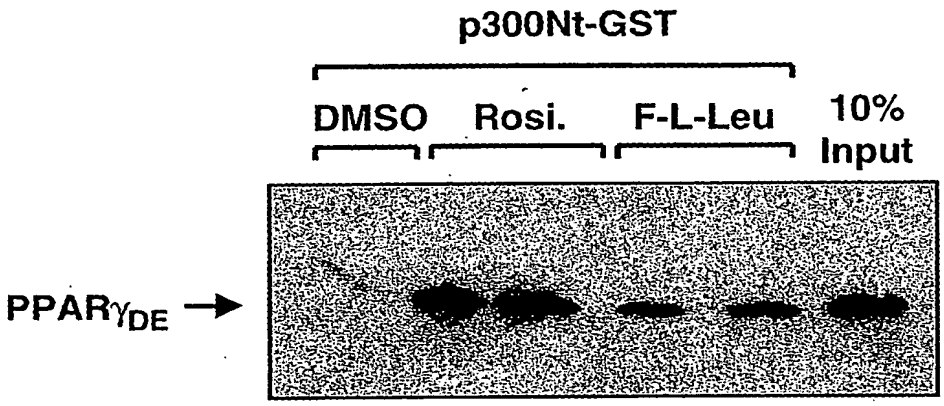


Figure 6

Figure 7A

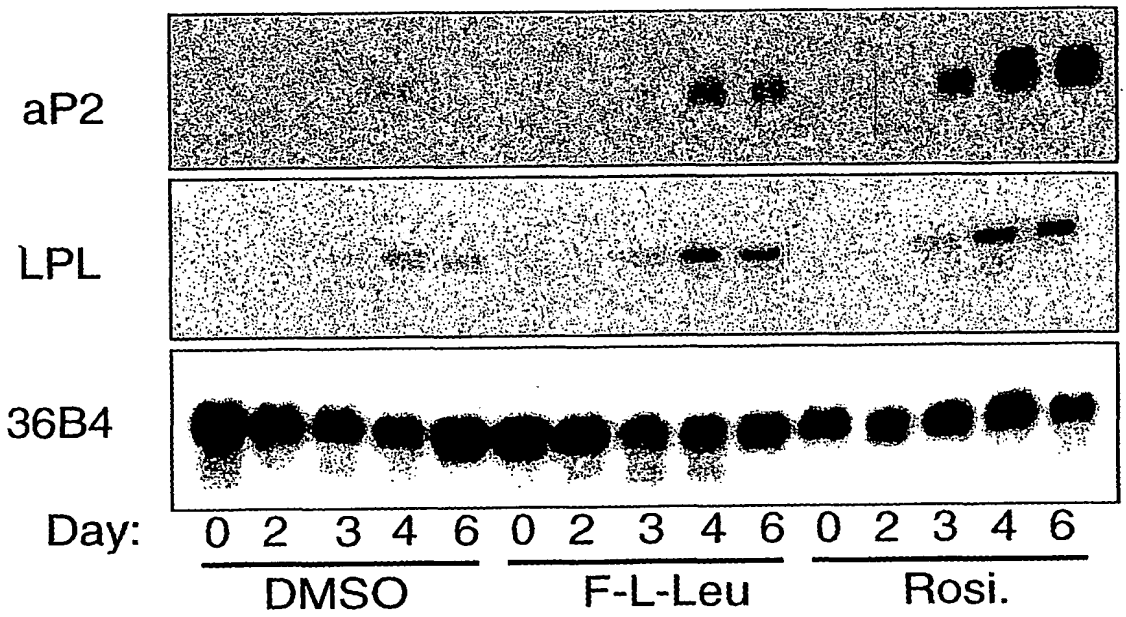


Figure 7B

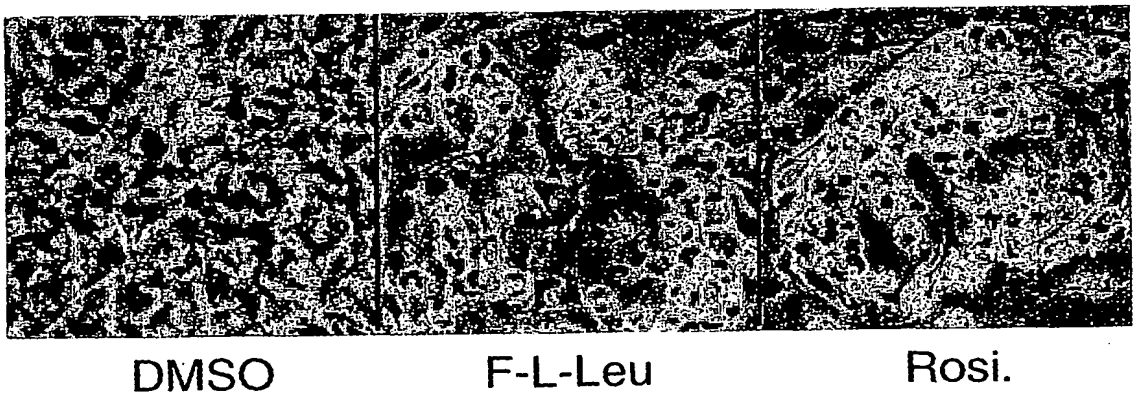


Figure 8A

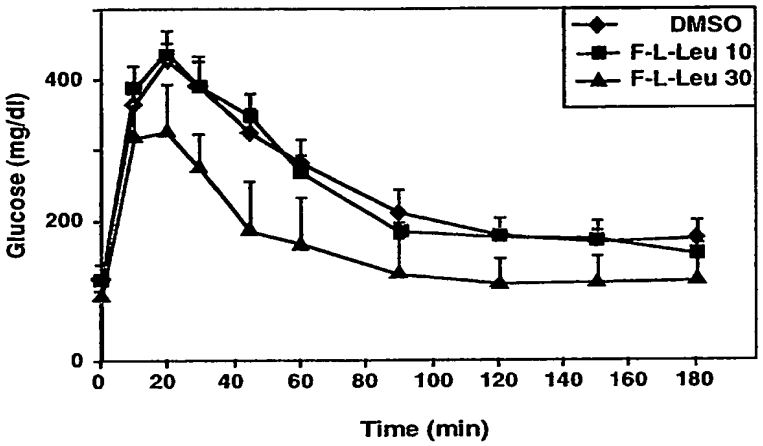


Figure 8B

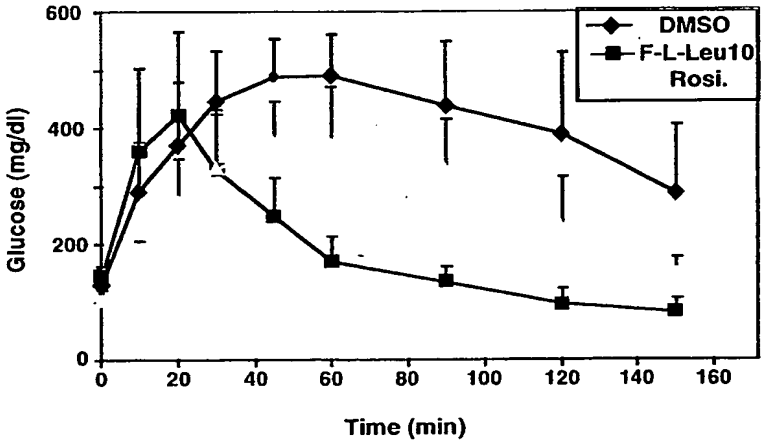


Figure 8C

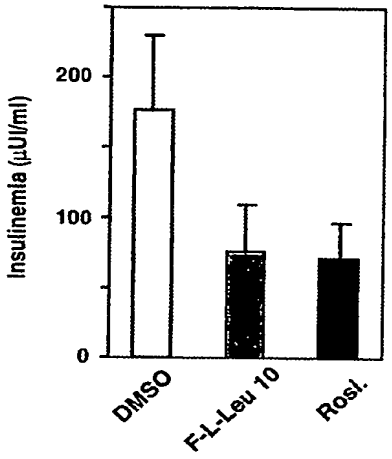


Figure 8D

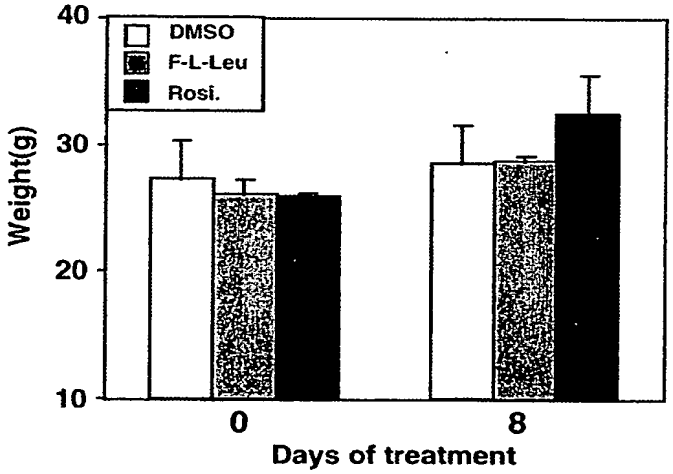


Figure 9A

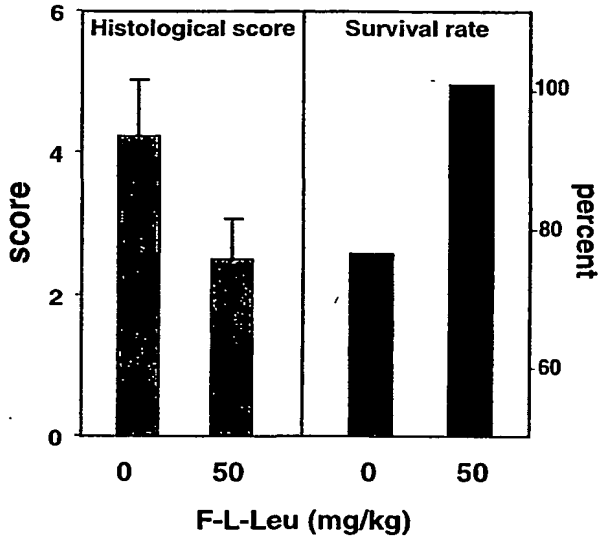
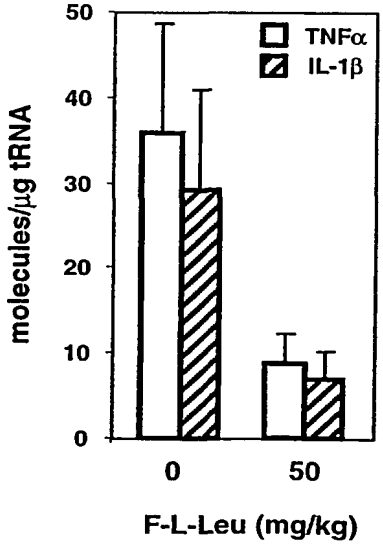


Figure 9B



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(10) International Publication Number
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(51) International Patent Classification[7]: C07C 271/22, A61K 31/325, A61P 7/00, 29/00, 1/00, 19/00, 35/00

(21) International Application Number: PCT/IB01/01581

(22) International Filing Date: 28 June 2001 (28.06.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/214,924 29 June 2000 (29.06.2000) US

(71) Applicant *(for all designated States except US)*: **ASSOCIATION POUR LE DEVELOPPEMENT DE LA RECHERCHE EN GENETIQUE MOLECULAIRE (ADEREGEM)** [FR/FR]; 231, rue de Charenton, F-75012 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROCCHI, Stéphane** [FR/FR]; 238, rue de Colmar, Les Urbaines, F-67000 Strasbourg (FR). **AUSWERX, Johan** [BE/FR]; 195, rue du Moulin, F-67150 Hindisheim (FR). **VAMECQ, Joseph** [BE/BE]; 189, chaussée de Beaumont, B-7030 Saint-Symphorien (BE).

(74) Agents: **MARTIN, Jean-Jacques** et al.; Cabinet Regimbeau, 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
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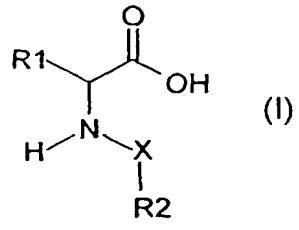
(54) Title: FMOC-L-LEUCINE AND DERIVATIVES THEREOF AS PPAR-GAMMA AGONISTS



(I)

(57) Abstract: The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine or derivatives thereof, of the formula I: Said method is particularly useful for treating or preventing anorexia, hyperlypidemia, insulin resistance, inflammatory diseases, cancer and sking disorders.

WO 02/00611 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 01/01581

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C271/22 A61K31/325 A61P7/00 A61P29/00 A61P1/00
A61P19/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 18596 A (WEITZBERG MOSHE ;BURCH RONALD MARTIN (US)) 12 December 1991 (1991-12-12) page 2, line 20 - line 26; claims 1-38; example 2	1-19

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2002	18/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Rufet, J

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-5, 9-19 all partly

Present claims 1-5,9-19 relate to an extremely large number of possible compounds used as drugs. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed (only one example). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds of claims 6 and 7.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ...ational Application No
PCT/IB 01/01581

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9118596 A	12-12-1991	US 5079260 A	07-01-1992
		AU 7951591 A	31-12-1991
		EP 0531443 A1	17-03-1993
		FI 925327 A	24-11-1992
		IE 911823 A1	04-12-1991
		JP 5509299 T	22-12-1993
		NZ 238272 A	25-03-1994
		WO 9118596 A1	12-12-1991
		ZA 9104102 A	24-02-1993

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(51) International Patent Classification[7]: C07C 271/22, A61K 31/325, A61P 7/00, 29/00, 1/00, 19/00, 35/00

(21) International Application Number: PCT/IB01/01581

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(71) Applicant *(for all designated States except US)*: **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)** [FR/FR]; 101, rue de Tolbiac, F-75013 Paris (FR).

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(75) Inventors/Applicants *(for US only)*: **ROCCHI, Stéphane** [FR/FR]; 238, rue de Colmar, Les Urbaines, F-67000 Strasbourg (FR). **AUWERX, Johan** [BE/FR]; 195, rue du Moulin, F-67150 Hindisheim (FR). **VAMECQ, Joseph** [BE/BE]; 189, chaussée de Beaumont, B-7030 Saint-Symphorien (BE).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(15) Information about Correction:
see PCT Gazette No. 20/2003 of 15 May 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/000611 A3

(54) Title: FMOC-L-LEUCINE AND DERIVATIVES THEREOF AS PPAR-GAMMA AGONISTS

(57) Abstract: The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine or derivatives thereof, of the formula I: Said method is particularly useful for treating or preventing anorexia, hyperlypidemia, insulin resistance, inflammatory diseases, cancer and sking disorders.

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International Bureau

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(51) International Patent Classification[7]: C07C 271/22, A61K 31/325, A61P 7/00, 29/00, 1/00, 19/00, 35/00

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(71) Applicant *(for all designated States except US)*: **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)** [FR/FR]; 101, rue de Tolbiac, F-75013 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROCCHI, Stéphane** [FR/FR]; 238, rue de Colmar, Les Urbaines, F-67000 Strasbourg (FR). **AUWERX, Johan** [BE/FR]; 195, rue du Moulin, F-67150 Hindisheim (FR). **VAMECQ, Joseph** [BE/BE]; 189, chaussée de Beaumont, B-7030 Saint-Symphorien (BE).

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(15) Information about Corrections:
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Previous Correction:
see PCT Gazette No. 20/2003 of 15 May 2003, Section II

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(54) Title: FMOC-L-LEUCINE AND DERIVATIVES THEREOF AS PPAR-GAMMA AGONISTS

(57) Abstract: The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine or derivatives thereof, of the formula I: Said method is particularly useful for treating or preventing anorexia, hyperlypidemia, insulin resistance, inflammatory diseases, cancer and sking disorders.

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FMOC-L-LEUCINE AND DERIVATIVES THEREOF AS PPAR- GAMMA AGONISTS

The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of FMOC-L-Leucine (N-(9-fluroroenylmethyloxycarbonyl)-L-Leucine) or derivatives thereof.

The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors which bind DNA as heterodimers with the retinoid X receptor (RXR) and activate a number of target genes, mainly involved in the control of lipid metabolism. PPARs have pleiotropic biological activities and wide-ranging medical applications, ranging from uses in metabolic disorders to eventual applications in inflammation, and cancer (Desvergne and Wahli, 1999; Schoonjans *et al.*, 1997; Spiegelman and Flier, 1996). Especially, PPARγ has received a lot of attention because PPARγ-activating drugs represent a novel opportunity to treat type 2 diabetes. PPARγ can be activated by naturally occurring ligands, such as the long-chain fatty acid-derivatives, 15-deoxy-Δ12,14-prostaglandin J2, Δ12-prostaglandin J2 (PG J2), and 9- and 13-cis-hydroxyoctadecadienoic acid (HODE) (Forman *et al.*, 1995; Kliewer *et al.*, 1995; Nagy *et al.*, 1998). Most interesting is, however, the observation that the anti-diabetic activity of a group of the glitazones, which all possess a thiazolidinedione ring (Figure 1, Panel A), results from their PPARγ activating properties (Berger *et al.*, 1996; Willson *et al.*, 1996). The therapeutic efficacy of the current thiazolidinediones (TZDs) in type 2 diabetes is, however, far from optimal and several undesirable side-effects have been reported for this drug class (Schoonjans and Auwerx, 2000). As a result, there is a need for non-TZD-based alternative ligands of PPARγ.

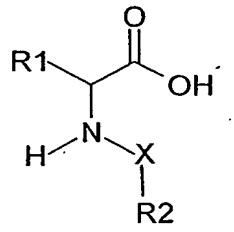
Recently, a series of L-tyrosine based PPARγ ligands were designed by replacing the thiazolidinedione ring with a carboxylic acid and by introducing an amine function on the adjacent carbon while keeping the parahydroxybenzyl sequence (Figure 1, Design 1 route). An optimal PPARγ activity was obtained when the amine on the alpha carbon of the L-tyrosine ligands was substituted with a benzoylphenyl function, leading to the development of *N*-(2-benzoylphenyl)-L-tyrosine derivatives (Figure 1, Panel B, left part) (Cobb *et al.*, 1998; Collins *et al.*, 1998). Rigidifying the benzoyl and phenyl moieties of this alpha-amino substituent through an additional phenyl-phenyl bond (Figure 1, Panel B, right part) leads to compounds with good potencies (Cobb *et al.*, 1998; Collins *et al.*, 1998).

In connection with the present invention, it was unexpectedly found that FMOC-L-tyrosine derivatives were devoid of PPARγ activity, whereas FMOC-L-leucine (hereafter also designated as F-L-Leu), whose structure is lacking the parahydroybenzyl sequence present in both the TZDs and previously developed L-tyrosine-based PPARγ ligands (Figure 1), is a new potent insulin-sensitizing compound with unique PPARγ-activating and -binding properties.

F-L-Leu, referred to as NPC 15199, has been described as a drug active in various inflammatory models through an unknown anti-inflammatory mechanism (Miller *et al.*, 1993) (Burch *et al.*, 1991). But, the present invention provides new applications of this compound and derivatives thereof as a PPARγ agonist.

Description

The present invention relates to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of a compound having the formula I:



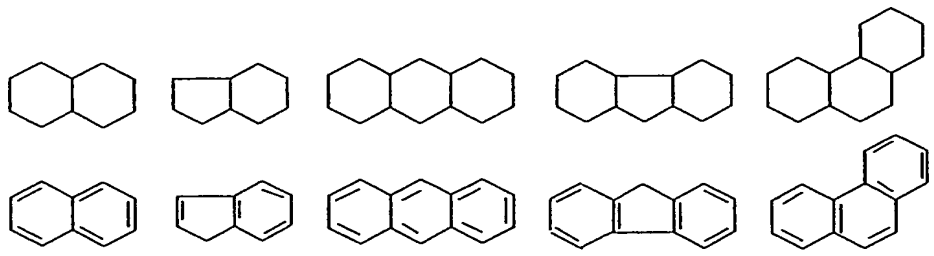
wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

X is a chain comprising from 1 to 6 carbon atoms which may comprise one to four heteroatoms,

R2 is a condensed polycyclic group comprising at least two cycles.

In a first embodiment, the R2 group comprises at least two cycles selected from carbocycles and heterocycles.

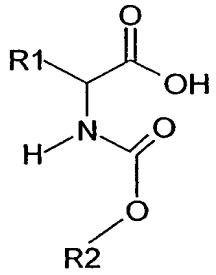
The R2 group can be advantageously selected from



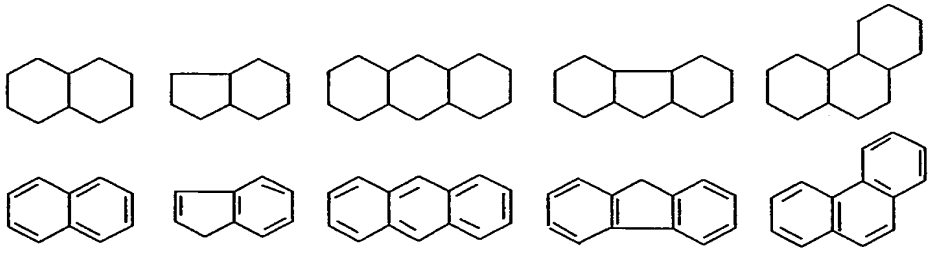
wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

In a second embodiment, the X chain comprises one or two carbon atoms which may be subtituted by an oxo group.

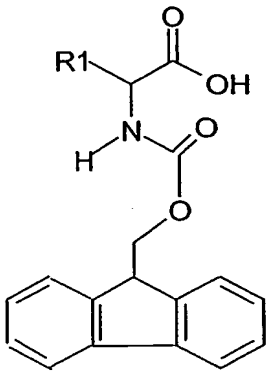
A preferred embodiment of the invention is directed to a method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,
R2 is a polycyclic group selected from

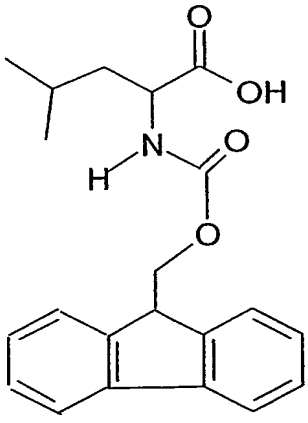


wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.
Compounds that are more particularly suitable have the formula :



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms and wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S.

For example, a preferred compound is



wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S; such as N-(9-fluroroenylmethyloxycarbonyl)-L-Leucine.

The method according to the invention is useful for treating or preventing anorexia, for increasing or decreasing body weight, treating or preventing hyperlypidemia, for increasing insulin sensitivity and for treating or preventing insulin resistance, as occurs in diabetes.

Among the other diseases or conditions that can be treated or prevented with the compounds described above, chronic inflammatory disorders such as inflammatory bowel disease, ulcerative colitis, Crohn's disease, arthritis, notably rheumatoid arthritis, polyarthritis and asthma are relevant.

The invention can also be reduced to practice for cancer, notably colon, prostate and hematological cancer, as well as for atherosclerosis and skin disorders, notably psoriasis.

We tested a number of the FMOC-aminoacid series for PPARγ-binding and activation. Interestingly, whereas FMOC-L-tyrosine, which was structurally most similar to the L-tyrosine based PPARγ ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998), was devoid of PPARγ-activating properties, another member of the FMOC-aminoacid series, F-L-Leu bound and activated PPARγ in a comprehensive set of *in vitro* and *in vivo* tests. Evidence supporting FMOC-L-leucine as a stereoselective PPARγ agonist ligand is provided by the following arguments:

1) F-L-Leu binds *in vitro* to PPARγ as evidenced by ESI-mass spectrometry (figure 5) and protease protection assays (figure 4);
2) F-L-Leu enhanced co-activators recruitment to the PPARγ protein (figure 6);
3) F-L-Leu activates PPARγ in cotransfection studies (figure 3);
4) F-L-Leu induces adipocyte differentiation as judged by increased lipid accumulation and the induction of adipocyte target genes, such as LPL and aP2 (figure 7);

5) F-L-Leu acts as a potent insulin-sensitizing agent in both diabetic and more interestingly also in non-diabetic murine models (figure 8);

6) Finally, like the TZDs ((Jiang *et al.*, 1998; Su *et al.*, 1999), F-L-Leu also had significant anti-inflammatory activities and could prevent inflammatory bowel disease (figure 9). Since F-L-Leu is clearly structurally different from thiazolidinediones and L-tyrosine based PPARγ ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998) and since F-L-Leu presents little or no structural analogies with the partial agonists GW0072 (Oberfield *et al.*, 1999) and L-764406 (Elbrecht *et al.*, 1999) and the antagonist BADGE (bisphenol A diglycidyl ether) (Wright *et al.*, 2000), F-L-Leu defines a chemically new class of PPARγ ligands.

Although F-L-Leu shares several functional characteristics with known PPARγ ligands, an important number of features distinguish F-L-Leu from these compounds, which will be addressed hereinafter.

F-L-Leu possesses an acidic function with the ability to liberate a proton, provided by its carboxylic group. This is a feature shared by the natural ligand, PG J2, as well as previously developed L-tyrosine based ligands. Such an acidic function is also present in the TZD ring at the level of the nitrogen located between the two carbonyl groups. A carboxylic group is also recovered in other PPARγ ligands such as GW0072, a weak partial agonist which antagonizes adipocyte differentiation, but in which lateral side-chain substitution is approximately ten carbon atoms distant from the carboxylate (Oberfield *et al.*, 1999). This distance is in contrast with agonists such as the tyrosine-derived ligands and F-L-Leu where a side-chain substitution occurs on the alpha-amino position. The stereoselectivity of the activation of PPARγ with the FMOC L- and D-leucines (L- by far more potent than the D-enantiomer) confirms previous observations

made on other ligands with an asymmetric carbon including 8-HETE (S>R) and an alpha-trifluoroethoxy-propanoic acid derivative (S>R) (Rangwala *et al.*, 1997; Young *et al.*, 1998; Yu *et al.*, 1995).

Experimental evidence suggests that F-L-Leu would interact with the ligand binding site of PPARγ in a fashion distinct from both the TZDs and the tyrosine-based ligands. Nuclear receptors generally only will dock one ligand molecule in their ligand binding pocket, which fits rather tightly around the ligand. Binding of both TZDs and tyrosine-based PPARγ ligands follows this paradigm of "1 ligand/1 receptor" (Willson *et al.*, 2000). The rather spacious ligand binding pocket of PPARγ, however, would not only allow the binding of large ligands, such as the tyrosine-based ligands, but eventually also allow binding of multiple ligand molecules to a single receptor. Our ESI-mass spectrometry data confirm that this is in fact the case with the F-L-Leu, where two molecules are shown to be bound to PPARγ ligand binding domain.

The capacity of PPARγ to bind two molecules of F-L-Leu could underly in fact some of the particular biological characteristics of this ligand. In fact, in transfection experiments F-L-Leu was two orders of magnitude less potent than the TZD, rosiglitazone, although both compounds had similar maximal efficacy. The lower potency and the steep dose-response curve could therefore be explained by the fact that a higher molar ratio of ligand to receptor is required to change its configuration, a finding consistent both with the results of our protease protection (figure 4) and cofactor interaction assays (figure 6).

Although, less potent *in vitro*, F-L-Leu compares rather favorably to TZDs, such as rosiglitazone, for anti-diabetic activity *in vivo*. Administration of F-L-Leu (10

mg/kg/day) to the diabetic db/db mice improved insulin sensitivity more dramatically than an equivalent dose of rosiglitazone. This could be deduced from the more robust reduction of the AUC in IPGTT for an almost equivalent reduction in fasting insulin levels. Furthermore, at a dose of 30 mg/kg/day F-L-Leu was able to significantly improve insulin sensitivity in normal animals, an effect never observed with glitazones. One caveat with this comparison between the *in vivo* efficacy of F-L-Leu relative to the TZDs, lies in the intraperitoneal route of drug administration used here. This is the only route described at present to be effective for F-L-Leu, but it is known to be suboptimal for TZDs, which are readily orally bioavailable. Despite this potential draw-back, the results obtained with F-L-Leu as a potential anti-diabetic drug remain, however, remarkable. Moreover, F-L-Leu structure does not share a TZD ring, but offers a isosteric version of this chemical group via a carboxylic function (see figure 1), which is devoid of TZD-related side effects.

In summary, we describe F-L-Leu as a small synthetic PPARγ ligand. Unlike known PPARγ ligands, two molecules of F-L-Leu bind to a single PPARγ molecule, making its mode of receptor interaction novel and interesting. This unique way of receptor interaction, underlies some of the particular pharmacological properties of F-L-Leu. In general, F-L-Leu exerts similar biological activities as the known groups of PPARγ agonists, with a distinct pharmacology, characterized by a lower potency, but similar maximal efficacy. This novel synthetic molecule represents hence a new pharmacophore, which can be optimized according to routine procedures, for modulation of PPARγ biological activity.

Figure legends

Figure 1: Schematic representation of PPARγ ligand structures. The different routes followed for the design are indicated.

A. anti-diabetic glitazones

B. L-tyrosine based PPARγ ligands

C. FMOC amino acids

Figure 2: Modulation of transcriptional activity of PPARγ2 by FMOC-amino-acid in Hep G2 cells. Hep G2 cells were co-transfected with an expression vector for PPARγ2 (0.1 μg/well), pGL3-$(J_{wt})_3$TKLuc reporter construct (0.5 μg/well), and pCMV-βGal (0.5 μg/well), as a control of transfection efficiency (0.5 μg/well). They were then grown during 24 h in the presence or absence of indicated compound. Activation is expressed as relative luciferase activity/β-galactosidase activity. Each point was performed in triplicate. This figure is representative of three independent experiments.

Figure 3: F-L-Leu enhances transcriptional activity of PPARγ2 in different cell lines. RK13 cells **(A and D)**, CV1 cells **(B)** or Hep G2 cells **(C)** were co-transfected with an expression vector for PPARγ2 (0.1 μg/well), pGL3-$(J_{wt})_3$TKLuc reporter construct (0.5 μg/well), and pCMV-βGal (0.5 μg/well), as a control of transfection efficiency (0.5 μg/well). They were then grown during 24 h in the presence or absence of indicated compound. Activation is expressed as relative luciferase activity/β-galactosidase activity. Each point was performed in triplicate, and each figure is representative of four independent experiments.

Figure 4: F-L-Leu ligand alters the conformation of PPARγ. ^{35}S-PPARγ was synthesized *in vitro* in a coupled transcription/translation system. Labeled PPARγ was subsequently incubated with DMSO (0.1%), rosiglitazone (10^{-4}M) or F-L-Leu (10^{-4}M),

followed by incubation with distilled water or increasing concentrations of trypsin. Digestion products were analyzed by SDS-PAGE followed by autoradiography. The migration of intact PPARγ is indicated and the asterisk indicates the 25-kDa resistant fragment of PPARγ.

Figure 5: Two molecules of F-L-Leu bind to a single PPARγ molecule. ESI-mass spectrometry analysis.

Figure 6: F-L-Leu enhances the interaction of PPARγ with p300. The purified his-tagPPARγ2$_{DE203-477}$ protein was incubated with purified p300Nt-GST protein and glutathione-Q-Sepharose beads in presence of DMSO (0.1%), rosiglitazone (10^{-4} M) or F-L-Leu (10^{-3} M). The beads were then washed and the samples separated on SDS-PAGE and blotted. The blot was developed with anti-histidine antibodies.

Figure 7: F-L-Leu enhances adipocyte differentiation. Confluent 3T3-L1 cells were incubated with 2 μM insulin, 1 μM dexamethasone, and 0.25 mM isobuthyl methyl xanthine for two days. Then, the cells were incubated in presence of DMSO (0.1 %), F-L-Leu (10^{-5} M) or rosiglitazone (10^{-7} M) for 4 days. **A:** RNA was isolated from 3T3-L1 cells after different times of differentiation induction. Blots were hybridized with 36B4 (to control for RNA loading); LPL or aP2 cDNAs. **B:** Cells were stained with Oil Red O after 6 days. LPL: lipoprotein lipase.

Figure 8: F-L-Leu improves insulin sensitivity in C57BL/6j and db/db mice. Intraperitoneal glucose tolerance test (IPGTT) in C57BL/6j **(A)** or db/db **(B)** mice 10 to 12 weeks old (n=8). Diamonds correspond to DMSO-treated mice; squares to F-L-Leu-treated mice at the concentration of 10 mg/kg/day and triangles to F-L-Leu-treated mice at the concentration of 30 mg/kg/day (for C57BL/6j mice, A) or rosiglitazone-treated mice at the concentration of 10 mg/kg/day (for db/db mice, B). Insulinemia **(C)** and body weights **(D)** of db/db mice treated with DMSO, F-L-Leu (10 mg/kg/day) or rosiglitazone (10 mg/kg/day).

Figure 9: F-L-Leu protects against colon inflammation in TNBS-treated Balb/c mice. A: Ameho histologic scores (left panel) and survival rate (right panel) in TNBS-treated mice injected either with DMSO or F-L-Leu (50 mg/kg/day). **B:** TNFα and IL-1β mRNA levels in the colon of TNBS-treated mice injected with DMSO or F-L-Leu (50 mg/kg/day). Results are expressed as mean ± SEM.

The following materials and methods were used to perform the examples below.

Materials and methods

FMOC-derivatives were acquired at Sen Chemicals (Dielsdorf, Switzerland). Rosiglitazone and pioglitazone were kind gifts of Dr. R. Heyman (Ligand Pharmaceuticals, San Diego, CA). The antibodies directed against the AB domain of PPARγ were produced in our laboratory (Fajas *et al.*, 1997). The protease inhibitor cocktail was purchased at ICN (Orsay, France).

Cell culture and transient transfection assays

The CV1, RK-13, and Hep G2 cell lines were obtained from ATCC (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics. Transfections with chloramphenicol acetyltransferase (CAT) or luciferase (luc.) reporter constructs were carried out exactly as described previously (Schoonjans *et al.*, 1996). The pGL3-$(J_{wt})_3$TKLuc and the pGL3-$(J_{wt})_3$TKCAT reporter constructs contain both three tandem repeats of the J site of the apolipoprotein A-II promoter cloned upstream of the herpes simplex virus thymidine kinase (TK) promoter and the luciferase or the CAT reporter genes respectively (Vu-Dac *et al.*, 1995). The following

expression vectors were used; pSG5-hPPARγ2, a construct containing the entire cDNA of the human PPARγ2 (hPPARγ2) (Fajas *et al.*, 1997); pSG5-mPPARα (Isseman *et al.*, 1993); and pCMV-βGal, as a control of transfection efficiency.

Production of proteins and mass spectrometry

The p300Nt-GST, fusion protein was generated by cloning the N-terminal part of the p300 protein (a.a. 2 to 516) downstream of the glutathione-S-transferase (GST) protein in the pGex-T1 vector (Pharmacia, Orsay, France). The fusion proteins were then expressed in *Escherichia coli* and purified on a glutathione affinity matrix (Pharmacia). Human PPARγ (aa. 203 to 477 of PPARγ) was subcloned into the pET15b (Novagen, Madison, WI) expression vector. The his-tagPPARγ2$_{DE203-477}$ proteins were produced as follow. The protein was purified using a metal chelate affinity column with an affinity column Co^{2+} coupled agarose (High Trap chelatin, Pharmacia). The protein was eluted with 20mM Tris-HCl, 500mM NaCl, 130mM imidazole and 1-2 propanediol 2.5% (pH 8.5). A second purification step was made by gel filtration (Superdex 200 16/60, Pharmacia). The protein was eluted with 20mM Tris-HCl, 100mM NaCl, 5mM DTT and 1-2 propanediol 2,5% (pH 8.5).

Liquid chromatography-electrospray ionization (ESI)-mass spectrometry analysis was performed as previously described (Rogniaux *et al.*, 1999).

Protease protection and pull-down experiments

Protease protection experiments. The pSG5-hPPARγ2 plasmid was used to synthesize ^{35}S-radiolabeled PPARγ in a coupled transcription/translation system according to the protocol of the manufacturer (Promega, Madison, WI). The transcription/translation reactions were subsequently aliquoted into 22.5 µl and 2,5 µl of phosphate buffered saline +/- compound were added. The mixture was separated into 4.5 µl aliquots and 0.5 µl of distilled water or distilled water-solubilized trypsin were added. The protease

digestion were allowed to proceed for 10 min at 25°C and terminated by the addition denaturing loading buffer. After separation of the digestion products in a gel SDS-PAGE 12% acrylamide, the gel was fixed in 10% acetic acid (v/v): 30% ethanol (v/v) for 30 min, treated in Amplify™ (Amersham, Orsay, France) and dried. The radiolabeled digestion products were visualized by autoradiography.

Pull-down experiments. The purified his-tagPPARγ DE proteins were incubated 1 hour at 22°C in pull-down buffer (phosphate-buffered saline 1x, Glycerol 10%, NP40 0,5%) with either GST or p300Nt-GST fusion protein, glutathione-Q sepharose beads, and F-L-Leu (10^{-3}M) or rosiglitazone (10^{-4}M) when necessary. The beads were then washed 4 times in pull-down buffer and boiled in 2x sample buffer. The samples were separated by 12% acrylamide SDS-PAGE and transferred to nitrocellulose membranes. Blots were developed with antibodies directed against polyhistidine aminoacid sequences.

Adipocyte differentiation

3T3-L1 cells (ATCC, Rockville, MD) were grown to confluence in medium A (Dulbecco's modified Eagle's Medium with 10% fetal calf serum, 100 units/ml penicillin, and 100µg/ml streptomycin). Confluent cells were incubated in medium A containing 2 µM insulin, 1 µM dexamethasone, and 0.25 mM isobuthyl methyl xanthine for two days. Then, the cells were incubated in medium A in presence or absence of PPARγ agonist for 4 days, changing the medium every 2 days. Adipogenesis was evaluated by analysis of the expression of adipocyte-specific markers and by staining of lipids with Oil Red O (Chawla and Lazar, 1994).

RNA preparation and analysis

RNA was isolated from 3T3-L1 cells by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Northern blot analysis of total cellular RNA was performed as described (Auwerx *et al.*, 1989). Lipoprotein lipase (LPL), aP2 and 36B4 were used as probes (Graves *et al.*, 1992; Laborda, 1991; Lefebvre *et al.*, 1997). For RT-competitive PCR, total RNA (5-10μg) was reverse transcribed into complementary DNA (cDNA) (Desreumaux *et al.*, 1999; Fajas *et al.*, 1997). The RT reaction mixture was amplified by PCR using sense and antisense primers specific for β-actin, TNFα and IL-1β. The samples were subjected to 40 PCR cycles, consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 52-58°C, and primer extension for 1.5 min at 72°C using a Gene Amp PCR System 9700 (Perkin-Elmer Corporation, Foster City, CA). The quantity of mRNA was expressed as the number of TNFα or IL-1β cDNA per β-actin cDNA molecules.

Animal experiments, glucose metabolism and inflammation

All mice were maintained in a temperature-controlled (25 °C) facility with a strict 12 h light/dark cycle and were given free access to food (standard mice chow; DO4, UAR, France) and water. Animals received F-L-Leu or rosiglitazone by intraperitoneal injection.

C57Bl/6J and db/db mice (8 per group) were obtained through the Janvier laboratories (Laval-Le Genest, France). Intraperitoneal glucose tolerance tests (IPGTT) were performed as described (Kaku *et al.*, 1988). Briefly, mice were fasted overnight (18h) and injected intraperitonealy (i.p.) with 25 % glucose in sterile saline (0.9 % NaCl) at a dose of 2 g glucose/kg body weight. Blood was subsequently collected from the tail for glucose quantification with the Maxi Kit Glucometer 4 (Bayer Diagnostic, Puteaux, France) prior to and at indicated times after injection. Blood for insulin measurement

was collected in fasting mice from the retroorbital sinus plexus under chloroform anesthesia. Plasma was separated and insulin measured using a radio immunoassay kit (Cis bio international, Gif-sur-Yvette, France).

Male Balb/c mice (8 per group) were used for the colitis studies (Jackson laboratories, Bar Harbor, Maine). Colitis was induced by administration of 40 µl of a solution of TNBS (150 mg/kg, Fluka, Saint Quentin Fallavier, France) dissolved in NaCl 0.9% and mixed with an equal volume of ethanol (50% ethanol). This solution was administered intrarectally via a 3.5 F catheter (Ref EO 3416-1, Biotrol, Chelles, France) inserted 4 cm proximal to the anus in anesthesized mice [Xylasine (50 mg/kg of Rompun® 2%, Bayer Pharma, Puteaux, France) and Ketamine (50 mg/kg of Imalgene® 1000, Rhône Mérieux, France)]. Animals were sacrified by cervical dislocation under ether anesthesia two days after TNBS administration. The colon was quickly removed, opened, washed. A 2 cm colonic specimen located precisely 2 cm above the anal canal was dissected systematically in 4 parts. One part was fixed overnight in 4% paraformaldehyde acid at 4°C, dehydrated in alcohol and embedded in paraffin. Sections (5 µm) were then deparaffined with xylene and rehydrated by ethanol treatment. Stained sections with haematoxylin-eosin were examined blindly by a pathologist and scored according to the Ameho criteria (Ameho *et al.*, 1997). The other parts of the colon were used for RNA isolation for the quantification of TNFα and IL1β mRNA expression.

Statistical analysis

Values were reported as mean +/- standard deviation. Statistical differences were determined by the Mann-Whitney U test. $P<0.05$ was accepted as statistically significant.

Example 1 : FMOC-L-leucine activates PPARγ in cell transfection experiments

Various FMOC derivatives of unsubstituted (L-tyrosine, D-leucine, and L-leucine) aminoacids were tested and compared to rosiglitazone or pioglitazone (as positive internal controls) for their ability to activate PPARγ in transient transfection experiments in HepG2 cells using the pSG5-hPPARγ2 expression and J_3TKpGl_3 reporter plasmids. In contrast to L-tyrosine PPARγ ligands (Cobb *et al.*, 1998; Collins *et al.*, 1998), the FMOC substituted L-tyrosine derivative did not activate PPARγ. Significant PPARγ activity could, however, be detected for F-L-leu at the concentration of 10^{-5} M (figure 2). In contrast, no significant PPARγ activation was detected with the FMOC-D-leucine derivative, demonstrating that PPARγ activating properties of F-L-leu were stereoselective. Additional transfection experiments with F-L-Leu were performed on different cell lines (RK13, CV1 and HepG2 cells) (figure 3 A, B and C). In the rabbit kidney RK13 cells, we found that rosiglitazone has an optimal activity between 10^{-8} to 10^{-7} M. For F-L-Leu, PPARγ activation occurred at concentrations of 10^{-5} M (figure 3A). Consistent with previous results, F-L-Leu concentrations of 10^{-5} M were also required for optimal PPARγ activation in simian renal cells CV1 (figure 3B), and in human HepG2 cells (figure 3C). The optimal concentration for PPARγ activation by F-L-Leu was similar to that of PG J2 and 100-fold higher than the concentration of rosiglitazone (figure 3C) or pioglitazone (data not shown) necessary to reach the same efficacy.

Finally, we tested whether FMOC-amino acid derivatives synergized or antagonized rosiglitazone activation of PPARγ in RK13 cells (figure 3D). No significant modification of PPARγ activity was observed when we added either F-L-Leu, FMOC L-tyrosine or FMOC D-leucine (10^{-5} M) to a saturating concentration of rosiglitazone.

These results furthermore confirmed (see figure 3A) that we reached maximal PPARγ activation using rosiglitazone and F-L-Leu at the concentration of 10^{-7}M and 10^{-5}M respectively.

Example 2: FMOC L-leucine changes PPARγ conformation

Thiazolidinediones can induce an alteration in the conformation of PPARγ, as assessed by generation of protease-resistant bands following partial trypsin digestion of recombinant receptor (Berger *et al.*, 1999; Elbrecht *et al.*, 1999). Upon incubation of rosiglitazone with PPARγ, a fragment of approximately 25 kDa is protected from trypsin digestion whereas no protection is detected when PPARγ is incubated with DMSO vehicle (figure 4). Interestingly, F-L-Leu produced a protease protection pattern similarly to rosiglitazone, demonstrating that F-L-Leu altered PPARγ conformation (figure 4).

Example 3: Two molecules of FMOC-L-leucine interact with PPARγ

Electrospray ionization (ESI) mass spectrometry of hPPARγ LBD (amino acid 203 to 477) was used to identify the specific binding of F-L-Leu with PPARγ (figure 5). The purified fragment of PPARγ LBD was incubated with vehicle alone or either 1 or 8 equivalents of F-L-Leu per equivalent of PPARγ. The mass of the receptor was determined after incubation by ESI-mass spectrometry. At 1 equivalent of F-L-Leu per equivalent of PPARγ, we could distinguish three populations of PPARγ corresponding to: 1/ unliganded PPARγ; 2/ a complex formed by 1 PPARγ LBD molecule and 1 F-L-Leu molecule; and 3/ a complex formed by 1 PPARγ LBD molecule and 2 F-L-Leu molecules. Interestingly, when we increased the F-L-Leu concentration (8 equivalents of F-L-Leu per 1 equivalent of PPARγ), we detected only the complex corresponding

to the PPARγ LBD bound with 2 F-L-Leu molecules. These results indicate that two molecules of F-L-Leu interact with one molecule of the PPARγ in a highly specific manner.

Example 4: FMOC-L-leucine enhances PPARγ/p300 interaction

PPARγ has been previously reported to interact with the cofactor p300. The overall molecular PPARγ/p300 interaction was the resultant of a ligand-independent binding of p300 to PPARγs' ABC domain and a ligand-dependent interaction of p300 with the PPARγ DE domains (Gelman *et al.*, 1999). Hence the purified PPARγ DE protein represents a tool to study the efficacy of PPARγ ligand binding properties in view of its' ability to recruit p300 upon ligand binding. Compared to the DMSO control, both rosiglitazone and F-L-Leu effectively induced the formation of PPARγ DE/p300Nt-GST complexes. This confirms that the F-L-Leu is a PPARγ ligand and that its' binding to the PPARγ DE domain is capable of inducing conformational changes required for association with p300. The potency of the F-L-Leu compound was in this assay 2- to 3-fold lower than that of rosiglitazone.

Example 5: FMOC-L-leucine induces adipocyte differentiation

The ability of F-L-Leu and rosiglitazone to stimulate adipocyte differentiation of murine pre-adipocyte 3T3-L1 cells were next compared. Adipogenesis was monitored by analysis of lipoprotein lipase (LPL) and aP2 mRNA levels as markers of adipocyte differentiation and by studying morphological changes associated with the differentiation process. F-L-Leu at the concentration of 10^{-5} M significantly stimulated both LPL and aP2 mRNA levels to an extent close to that seen in cells incubated with

rosiglitazone at the concentration of 10^{-7} M (figure 7A). Staining of 3T3-L1 cells with Oil Red O, as a marker for neutral lipid accumulation, was performed after a 6 days incubation of cells with either DMSO, or the two PPARγ ligands F-L-leu or rosiglitazone (Figure7B). The two drugs were again capable of inducing neutral lipid accumulation. Hence, like rosiglitazone, F-L-Leu was an adipogenic drug in 3T3-L1 cells.

Example 6: FMOC-L-leucine improves insulin sensitivity *in vivo*

To assess whether F-L-Leu could improve insulin sensitivity, we compared the glucose tolerance in C57BL/6j mice treated with F-L-Leu relative to that observed in control animals which received only the vehicle, DMSO (figure 8A). Mice were treated with 2 different doses of F-L-Leu (10 and 30 mg/kg/days) during 7 days and then IPGTT was performed. Intra-peritonealy administrated glucose was cleared in a comparable rate in mice receiving vehicle or F-L-Leu at 10 mg/kg/day. In mice treated with F-L-Leu at 30 mg/kg/day, the maximum glucose levels increased only to 320 mg/dl whereas the glucose levels climbed to 440 mg/dl after glucose injection for both 10 mg/kg/day F-L-Leu and the control group. Furthermore, the area under the curve was significantly lower in mice treated with F-L-Leu at 30 mg/kg/day relative to either control mice or mice receiving F-L-Leu at lower dose.

We next compared glucose tolerance in db/db mice treated with DMSO, F-L-Leu (10 mg/kg/day) or rosiglitazone (10 mg/kg/day) during 7 days. In control mice (DMSO group), glycemia rapidly increased after glucose loading, reaching a maximum of 500 mg/dl between 45 to 60 min after injection, before slowly decreasing. In rosiglitazone-treated mice, glucose loading was better "tolerated" than in control animals with a

reduction in the maximal glycemia (350 mg/dl), and a more rapid recovery of these supranormal values. F-L-Leu-treated animals showed the best glucose tolerance test, with a maximal glucose level (420 mg/dl) 20 min after injection and an immediate and fast subsequent decrease to normal (100 mg/dl) values within 120 min. Furthermore, 7 days treatment of animals with F-L-Leu and rosiglitazone resulted in a dose-dependent lowering of fasting serum insulin levels (mean values of 70 µUI/mL for db/db mice treated with either F-L-Leu or rosiglitazone versus 180 µUI/mL for the DMSO group) (figure 8C). These data clearly show that F-L-Leu improves insulin sensitivity in both diabetic and normal mice. Interestingly, whereas rosiglitazone had a tendency to increase body weight of mice, no difference in body weight was seen in mice treated with F-L-Leu during 8 days when compared to control mice (figure 8D). In addition, we observed a tendency to diminution of the liver weight for F-L-Leu-treated mice relative to control or rosiglitazone-treated mice (data not shown).

Example 7: FMOC-L-leucine protects against colitis

Intrarectal administration of TNBS has been shown to induce rapidly and reproducibly a colitis in mice as a result of covalent binding of TNP residues to autologuous host proteins leading to a mucosal infiltration by polynuclear cells, the production of TNFα , and the activation of NFκB (Allgayer *et al.*, 1989; Stenson *et al.*, 1992; Su *et al.*, 1999).We determined the survival rate and scored the colon damage as well as the production of cytokines two days after intra-rectal TNBS administration in control animals or animals which were treated 4 days with F-L-Leu at 50 mg/kg/day (figure 9). Interestingly, 100% of F-L-Leu-treated mice survived colon inflammation whereas only 76 % of control mice were alive after induction of inflammation. Administration of F-L-Leu furthermore reduced significantly the histologic score indicating that F-L-

Leu reduces ulceration, erosion and necrosis induced by inflammation. Finally, F-L-Leu administration resulted in a significant decrease in the mRNA levels expression of the pro-inflammatory cytokines, TNFα and IL-1β suggesting that, like with rosiglitazone, PPARγ activation by F-L-Leu protects against colon inflammation by inhibition of the TNFα signaling pathway.

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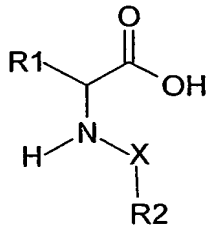
CLAIMS

1. A method for treating or preventing a PPAR-γ mediated disease or condition comprising administration of a therapeutically effective amount of a compound having the formula I:



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

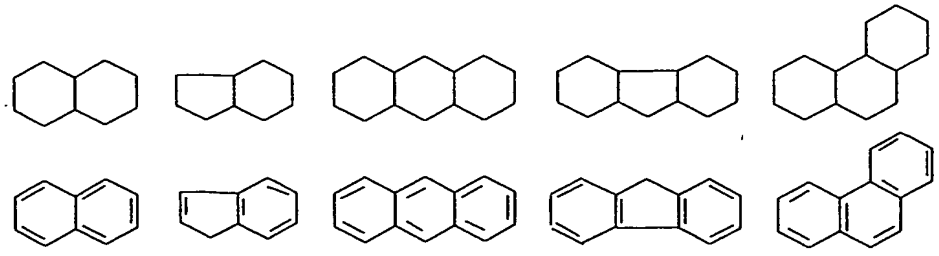
X is a chain comprising from 1 to 6 carbon atoms which may comprise one to four heteroatoms,

R2 is a condensed polycyclic group comprising at least two cycles.

2. A method according to claim 1 wherein the R2 group comprises at least two cycles selected from carbocycles and heterocycles.

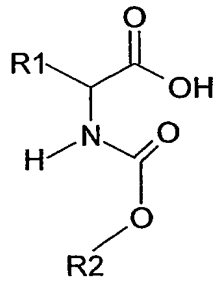
3. A method according to one of claims 1 and 2 wherein the X chain comprises one or two carbon atoms which may be substituted by an oxo group.

4. A method according to one of claims 1 to 3 wherein R2 is a polycyclic group selected from



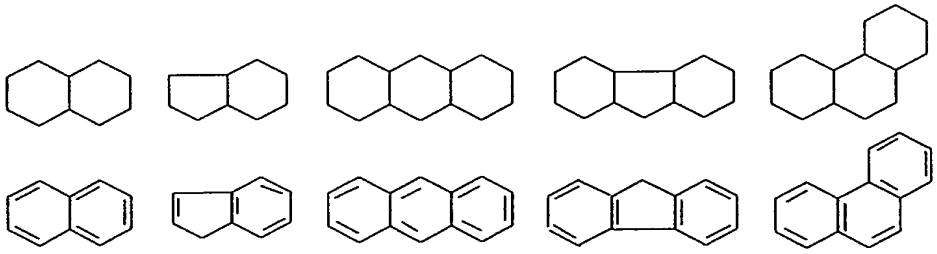
wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

5. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



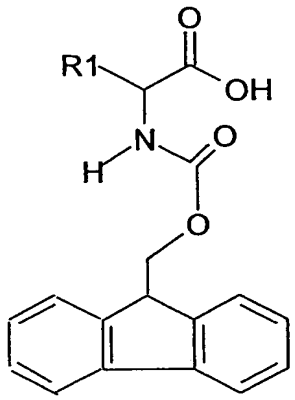
wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms,

R2 is a polycyclic group selected from



wherein said groups optionally comprise one to four heteroatoms selected from halogens, N, O and S.

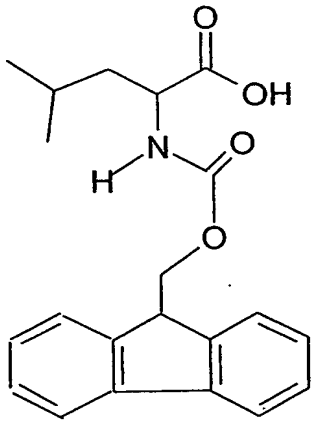
6. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein R1 is selected from a linear or branched alkyl, alkenyl and alkynyl group comprising from 1 to 6 carbon atoms and

wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S

7. A method according to claim 1 comprising administration of a therapeutically effective amount of a compound the formula I, wherein said compound is



wherein the said tricyclic group optionally comprises one to four heteroatoms selected from halogens, N, O and S

8. A method according to claim 1 comprising administration of a therapeutically effective amount of N-(9-fluroroenylmethyloxycarbonyl)-L-Leucine.

9. A method according to one of claims 1 to 8 wherein said disease or condition is anorexia.

10. A method according to one of claims 1 to 8 for increasing or decreasing body weight.

11. A method according to one of claims 1 to 8 for increasing insulin sensitivity.

12. A method according to one of claims 1 to 8 for treating or preventing insulin resistance, as occurs in diabetes.

13. A method according to one of claims 1 to 8 wherein said disease or condition is a chronic inflammatory disorder.

14. A method according to one of claims 1 to 8 wherein said disease or condition is inflammatory bowel disease, ulcerative colitis or Crohn's disease.

15. A method according to one of claims 1 to 8 wherein the said disease or condition is arthritis, notably rheumatoid arthritis, polyarthritis and asthma.

16. A method according to one of claims 1 to 8 wherein said disease is cancer.

17. A method according to one of claims 1 to 8 wherein said disease is atherosclerosis.

18. A method according to one of claims 1 to 8 wherein said disease is a skin disorder, notably psoriasis.

19. A method according to one of claims 1 to 8 wherein said disease is hyperlypidemia.

Figure 1B

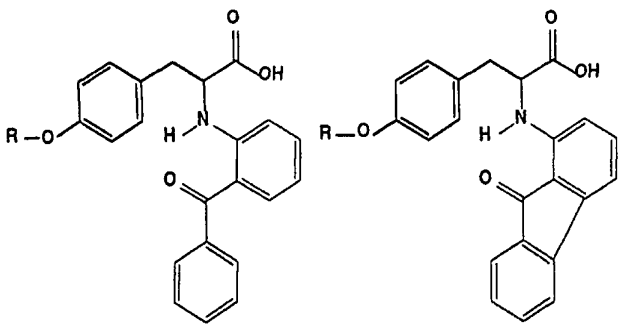
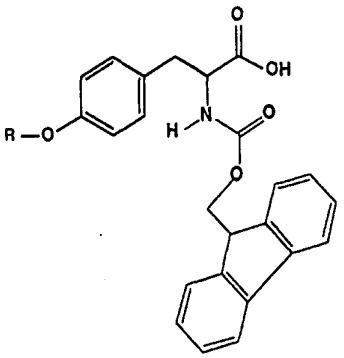
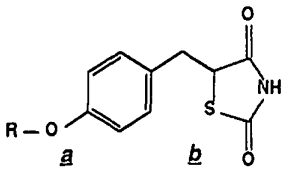


Figure 1C

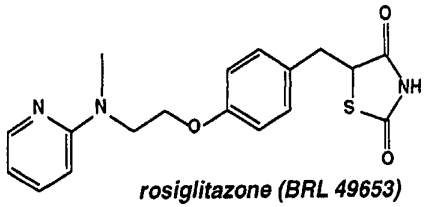


Design2

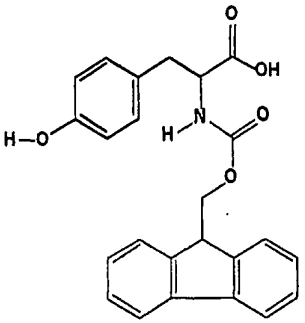
Design1



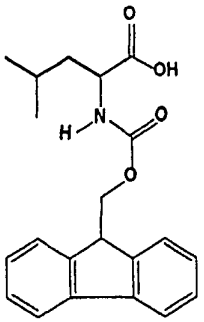
a b



rosiglitazone (BRL 49653)



FMOC-L-tyrosine



FMOC-L-leucine

Figure 1A

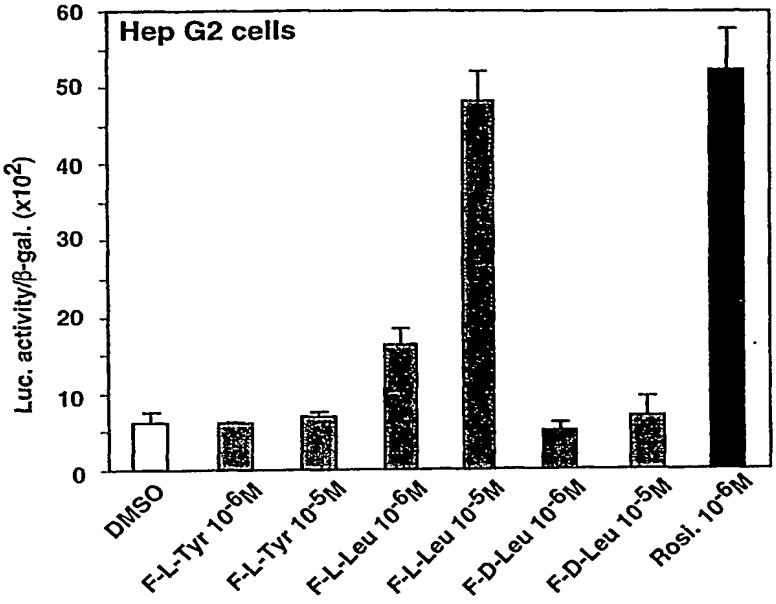

Hep G2 cells
Luc. activity/β-gal. (x10²)
60
50
40
30
20
10
0
DMSO
F-L-Tyr 10⁻⁶M
F-L-Tyr 10⁻⁵M
F-L-Leu 10⁻⁶M
F-L-Leu 10⁻⁵M
F-D-Leu 10⁻⁶M
F-D-Leu 10⁻⁵M
Rosi. 10⁻⁶M

Figure 2

Figure 3A

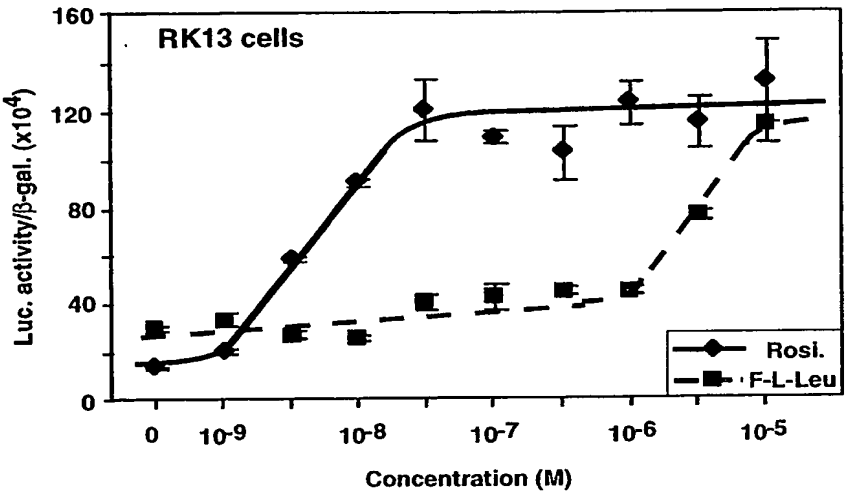


Figure 3B

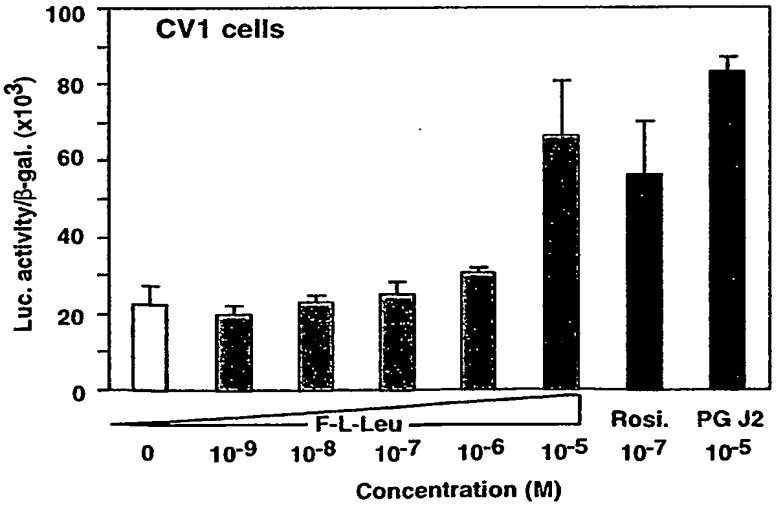


Figure 3C

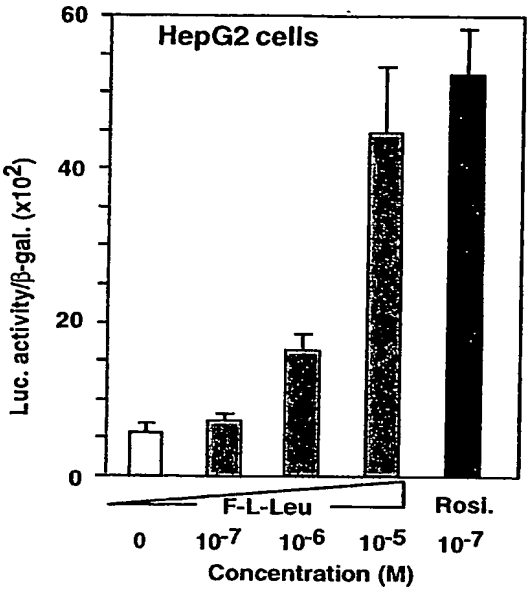
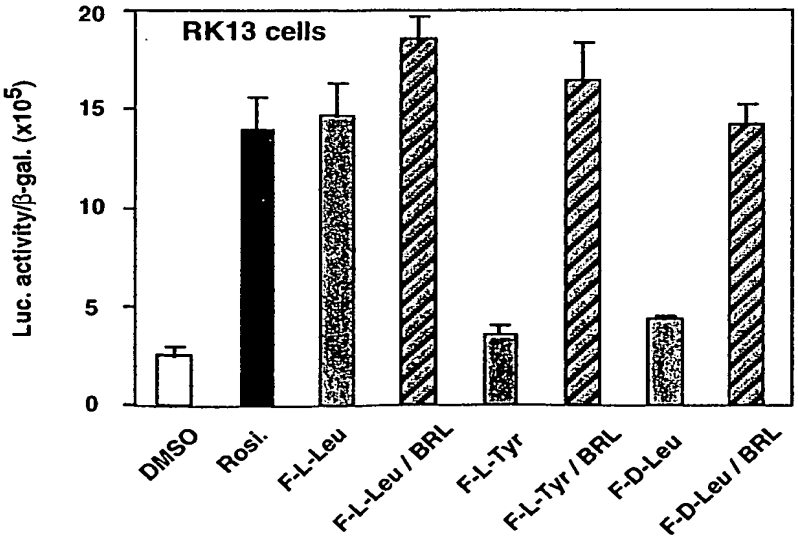


Figure 3D



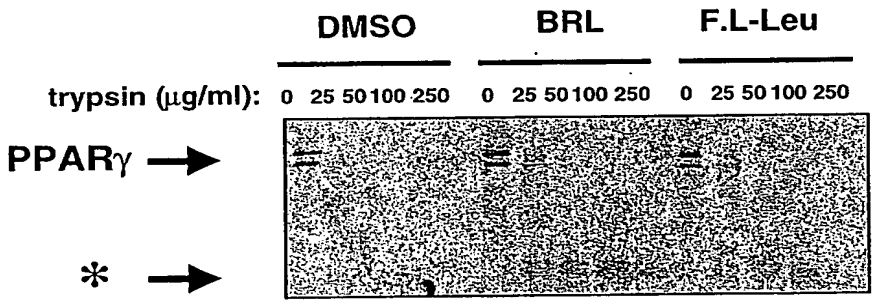

DMSO
BRL
F.L-Leu
trypsin (μg/ml): 0 25 50 100 250 0 25 50 100 250 0 25 50 100 250
PPARγ
*

Figure 4

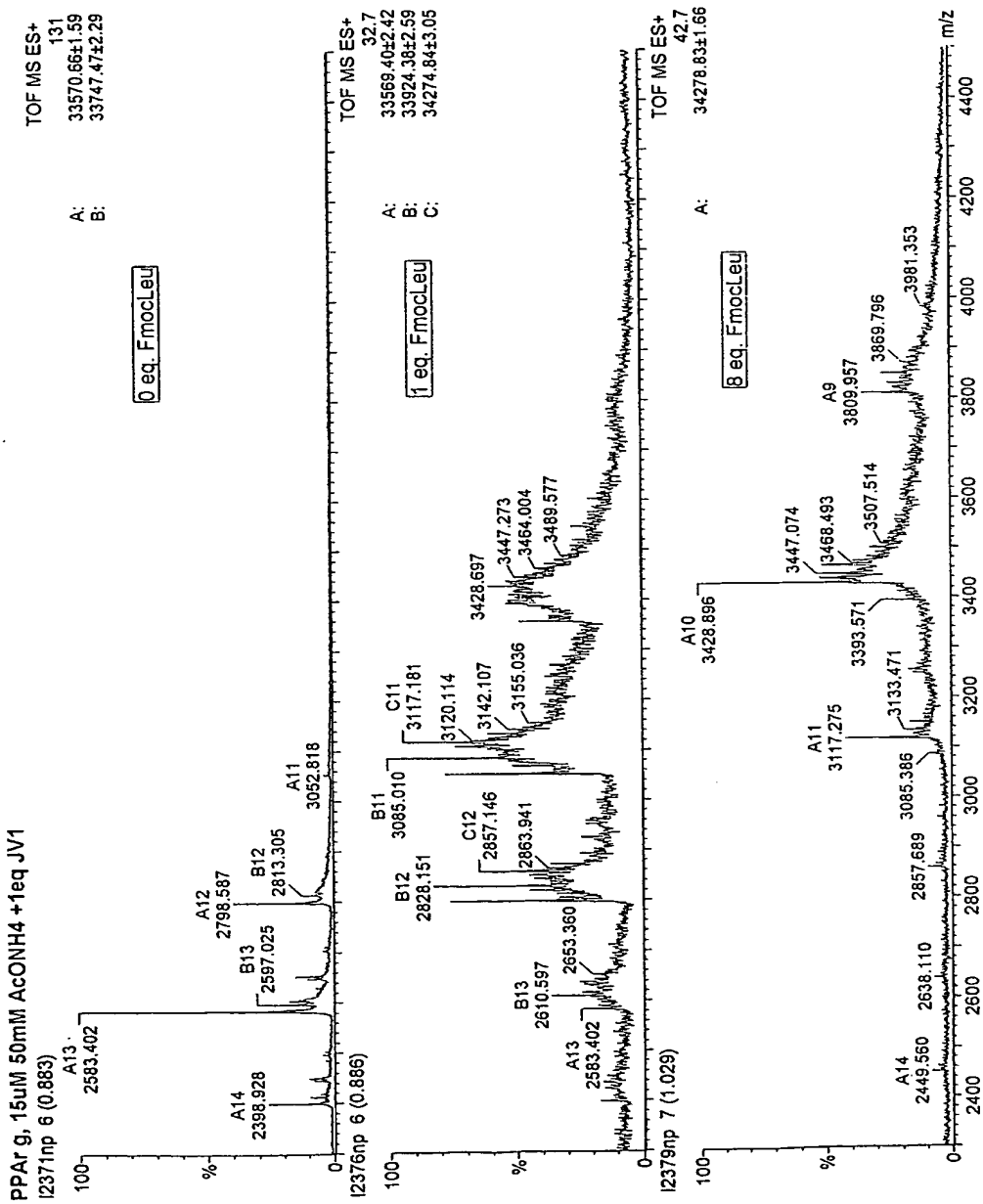

PPAr g, 15uM 50mM AcONH4 +1eq JV1
I2371np 6 (0.883)
I2376np 6 (0.886)
I2379np 7 (1.029)
0 eq. FmocLeu
1 eq. FmocLeu
8 eq. FmocLeu
TOF MS ES+
131
A: 33570.66±1.59
B: 33747.47±2.29
TOF MS ES+
32.7
A: 33569.40±2.42
B: 33924.38±2.59
C: 34274.84±3.05
TOF MS ES+
42.7
A: 34278.83±1.66
A13 2583.402
A14 2398.928
B13 2597.025
A12 2798.587
B12 2813.305
A11 3052.818
B13 2610.597
2653.360
A13 2583.402
B12 2828.151
C12 2857.146
2863.941
B11 3085.010
C11 3117.181
3120.114
3142.107
3155.036
3428.697
3447.273
3464.004
3489.577
A14 2449.560
2638.110
2857.689
3085.386
A11 3117.275
3133.471
3393.571
A10 3428.896
3447.074
3468.493
3507.514
A9 3809.957
3869.796
3981.353
%
100
0
2400
2600
2800
3000
3200
3400
3600
3800
4000
4200
4400
m/z

Figure 5

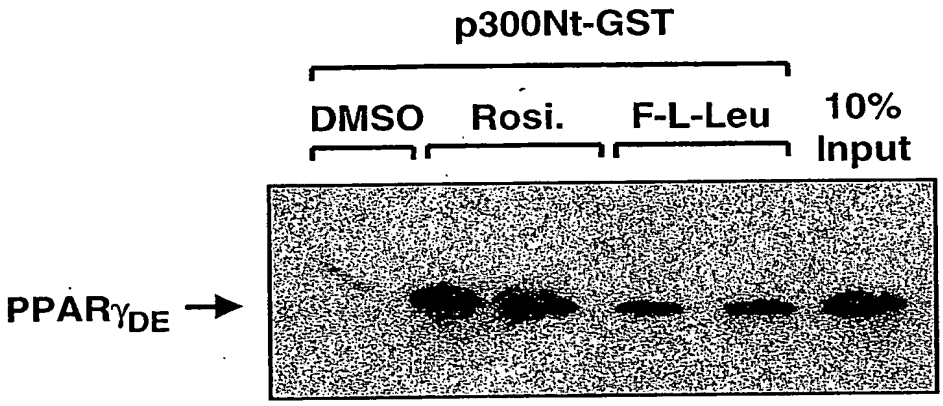


Figure 6

Figure 7A

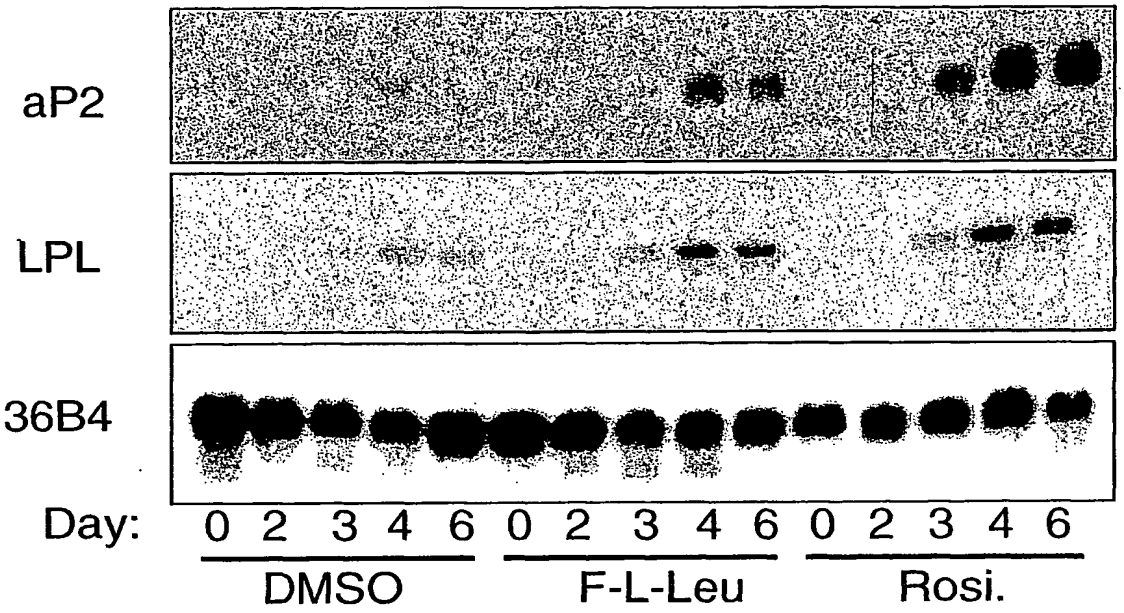


Figure 7B

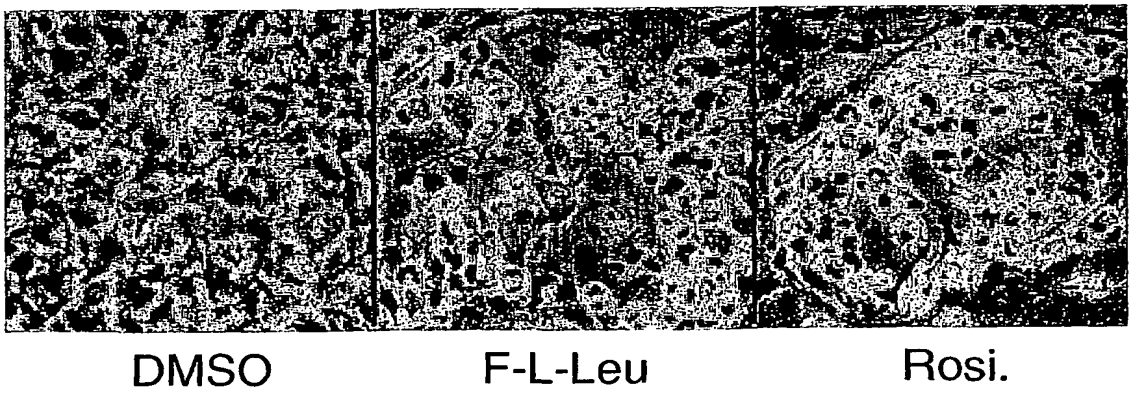


Figure 8A

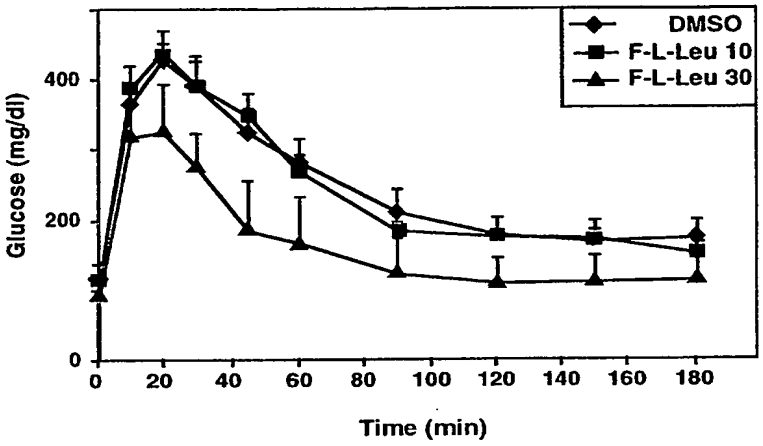


Figure 8B

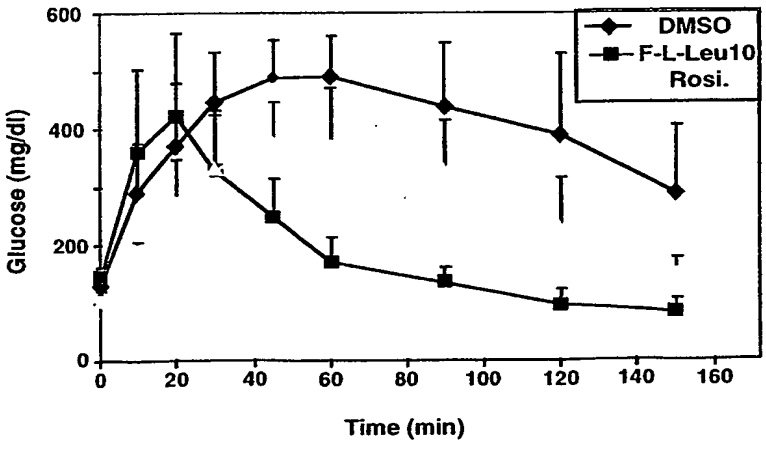


Figure 8C

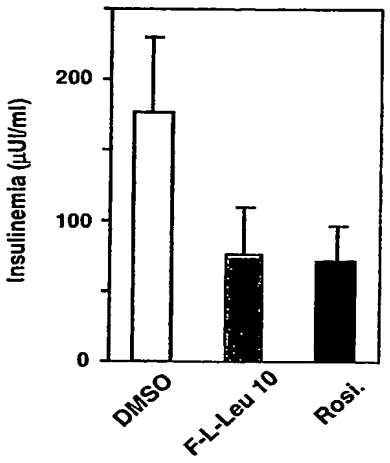


Figure 8D

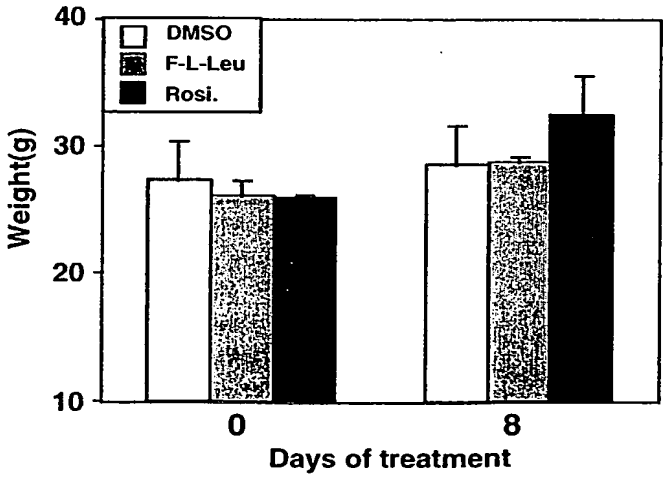


Figure 9A

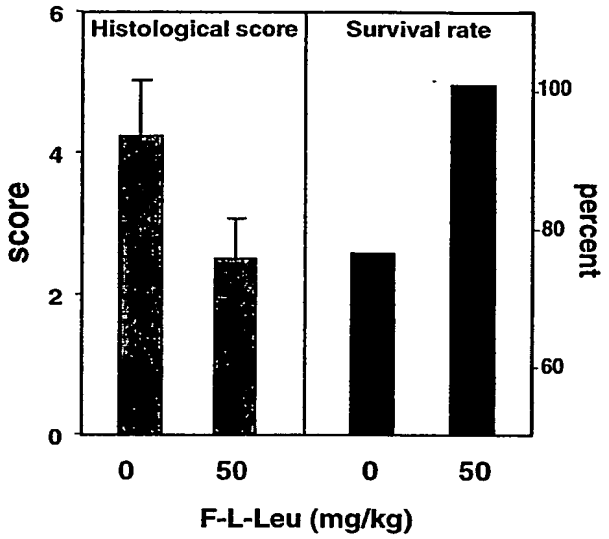
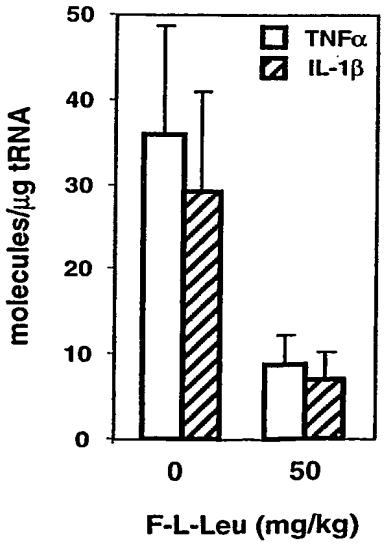


Figure 9B



INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 01/01581

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C271/22 A61K31/325 A61P7/00 A61P29/00 A61P1/00
A61P19/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 18596 A (WEITZBERG MOSHE ;BURCH RONALD MARTIN (US)) 12 December 1991 (1991-12-12) page 2, line 20 - line 26; claims 1-38; example 2	1-19

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2002	18/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Rufet, J

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-5, 9-19 all partly

Present claims 1-5,9-19 relate to an extremely large number of possible compounds used as drugs. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed (only one example). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds of claims 6 and 7.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 01/01581

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9118596 A	12-12-1991	US 5079260 A	07-01-1992
		AU 7951591 A	31-12-1991
		EP 0531443 A1	17-03-1993
		FI 925327 A	24-11-1992
		IE 911823 A1	04-12-1991
		JP 5509299 T	22-12-1993
		NZ 238272 A	25-03-1994
		WO 9118596 A1	12-12-1991
		ZA 9104102 A	24-02-1993